PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/53

A2

(11) International Publication Number: WO 00/60355

(43) International Publication Date: 12 October 2000 (12.10.00)

(21) International Application Number: PCT/US00/08841

(22) International Filing Date: 3 April 2000 (03.04.00)

(30) Priority Data:
09/285,477 2 April 1999 (02.04.99) US

(71) Applicants *(for all designated States except US)*: ICOS CORPORATION [US/US]; 22021 20th Avenue, S.E., Bothell, WA 98021 (US). ABBOTT LABORATORIES [US/US]; 100 Abbott Park Road, Abbott Park, IL 60064–6050 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: STAUNTON, Donald [US/US]; 6502 113th Avenue, N.E., Kirkland, WA 98033 (US). VAN DER VIEREN, Monica [US/US]; 2446 N.W. 64th Avenue, Seattle, WA 98107 (US). HUTH, Jeff [US/US]; 1103 Tracy Lane, Libertyville, IL 60048 (US). FOWLER, Kerry [US/US]; 747 North 66th Street, Seattle, WA 98103 (US). ORME, Mark [US/US]; 4235 Francis Avenue, #203, Seattle, WA 98103 (US). OLEJNICZAK, Edward, T. [US/US]; 506 Laurie Court, Grayslake, IL 60030 (US).

(74) Agent: WILLIAMS, Joseph, A., Jr.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606–6402 (US).

(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: LFA–1 REGULATORY BINDING SITE AND USES THEREOF

(57) Abstract

Methods to negatively regulate LFA–1 binding to an ICAM that binds LFA–1 are provided, in addition to a novel regulatory binding site on LFA–1.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

LFA-1 REGULATORY BINDING SITE AND USES THEREOF

BACKGROUND

The leukocyte function-associated antigen (LFA-1, CD11a/CD18) is a leukocyte-specific β_2 integrin that participates in cell/cell adhesion. Binding activity of LFA-1 is essential to leukocyte extravasation from circulation to a site of injury in an inflammatory response. Three principle ligands are known to bind LFA-1, ICAM-1, ICAM-2, and ICAM-3. These are intercellular adhesion molecules that play an important role in localizing leukocyte adhesion to endothelial cells at a site of injury. ICAM-4 and ICAM-5 have also been reported to bind LFA-1. Most leukocytes constitutively express LFA-1, but ligand binding requires activation believed to induce a conformational change and to increase avidity ligand binding. For example, ICAM-1 is normally expressed at low levels on the endothelium. However, injury-induced inflammatory mediators promote enhanced surface expression in cells at the site of the injury which, in turn, promotes localized leukocyte adhesion through binding to activated LFA-1.

The structure of LFA-1 includes distinct intracellular and extracellular domains that are believed to participate in and/or regulate ICAM binding. Of particular interest is a region in the α_L chain of approximately 200 amino acids, designated the I domain, that is found in all β_2 integrins, as well as many other proteins. Evidence suggests that the I domain is essential to LFA-1 binding to ICAM-1 and 3. For example, anti-LFA-1 blocking monoclonal antibodies have been mapped to epitopes within the I domain. In addition, recombinant I domain polypeptide fragments have been shown to inhibit integrin-mediated adhesion and bind ICAM-1. Within the I domain of LFA-1 (and other proteins) is a single metal ion dependent adhesion site (MIDAS) that preferentially binds manganese or magnesium ions. Binding of either cation is required for ligand interaction and is believed to induce conformational changes in LFA-1 necessary for binding. Cation binding may therefore be a regulatory mechanism that responds to changes in the extracellular leukocyte environment. This hypothesis is supported by the observation that calcium ion binding actually inhibits LFA-1 interaction with ICAM-1. Indeed, it has been

proposed that an inactive LFA-1 conformation results from calcium binding, and that replacement of the calcium ion with a manganese or magnesium ion is a step required for LFA-1 activation [Griggs, *et al., J. Biol. Chem. 273*:22113-22119 (1998)]. Other factors have also been shown to induce LFA-1 activation, including T cell receptor engagement, cytokine stimulation, and *in vitro* PMA stimulation.

In practical terms, the identification of LFA-1/ICAM binding sites provides targets to modulate leukocyte inflammatory responses. Numerous antibodies have been isolated that are capable of inducing LFA-1 activation [see, for example, Landis, *et al., J. Cell Biol. 120*:1519-1527 (1993)] or that are capable of preventing ICAM-1 interaction [see for example, Randi and Hogg, *J. Biol. Chem. 269*:12395-12398 (1994)]. The previous identification of anti-LFA-1 activating antibodies that recognize multiple and distinct extracellular epitopes suggests the existence of more than one regulatory region, presumably independent of cytoplasmic signaling. Localization of LFA-1 sites that bind ICAM-1 has been investigated through use of chimeric LFA-1 α subunit proteins comprising human and murine components [Huang and Springer, *J. Biol. Chem. 270*:19008-19016 (1995)]. Studies have indicated that residues that coordinate cation binding and residues proximal to the site are essential for binding ICAM-1 at a relatively flat interface. More precise delineation of the extracellular regulatory region(s) and the contact points for ICAM-1 binding will permit design of efficient modulators.

Thus there exists a need in the art to precisely identify regulatory regions for proteins that participate in inflammatory responses, and in particular LFA-1 and ICAMs that bind LFA-1. Determining the tertiary (or quaternary) structure of a protein can identify potential regulatory regions to permit the rational design of biologically compatible small molecules for therapeutic and prophylactic intervention for inflammatory disorders. There further exists a need in the art to identify compounds that can inhibit LFA-1 binding to ICAMs that can be used in the treatment of inflammatory disorders.

SUMMARY OF THE INVENTION

The present invention provides methods for identifying a negative regulator of LFA-1 binding to a natural ligand that competes for binding to LFA-1 with ICAM-1 or ICAM-3 comprising the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, and a ligand that binds LFA-1, or an LFA-1-binding fragment thereof, in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site presenting a diaryl sulfide binding conformation defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 as set out in SEQ ID NO: 2, which provides the amino acid sequence for mature (*i.e.*, without the leader sequence) LFA-1. "Natural ligand" refers to any biological compound that binds LFA-1. The term "negative regulator" refers to a compound that decreases ICAM binding to LFA-1, but does not directly compete with the ICAM for LFA-1 binding. A negative regulator may be an allosteric inhibitor or a compound that modulates the activation state of LFA-1. In a preferred method, the negative regulator is a diaryl sulfide. In a preferred embodiment, the natural ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

In another aspect, the invention provides methods for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, and a natural ligand that binds LFA-1, or an LFA-1-binding fragment thereof, in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ligand and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide. In a preferred method, the negative regulator is a diaryl sulfide. Preferably, the ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

The invention also provides screening methods for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising

the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide for binding to LFA-1 α_L polypeptide. In a preferred method, the negative regulator is a diaryl sulfide.

The invention also provides pharmaceutical compositions comprising a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 identified by a method of the invention. The invention further provides use of a negative regulator identified by a method of the invention in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to an ICAM that binds LFA-1.

The invention further provides methods for inhibiting LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of contacting LFA-1, or a ligand binding fragment thereof, with a negative regulator compound; said negative regulator binding the LFA-1 α_L polypeptide, or a fragment thereof, at a site selected from the group consisting of a conformation that binds a diaryl sulfide, a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 α_L polypeptide, and an LFA-1 domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide . In a preferred method, the negative regulator is a diaryl sulfide. In one embodiment, methods of the invention include use of cells expressing either LFA-1 or the ligand. In methods wherein one of the binding partners is expressed in a cell, the other binding partner is either purified and isolated, in a fluid sample (purified, partially purified, or crude) taken from an individual, or in a cell lysate. The invention also comprehends methods wherein both LFA-1 and the ICAM are expressed in cells. The LFA-1 and ligand binding partners may be expressed on the same cell type or different cell types.

The invention also provides methods to inhibit leukocyte adhesion to endothelial cells comprising the step of contacting said leukocyte with a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1, said negative regulator binding an LFA-1 regulatory site selected from the group consisting of a site

that binds a diaryl sulfide, a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 α_L polypeptide, and an LFA-1 domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide. *In vivo* and *in vitro* methods are contemplated. In a presently preferred embodiment, the negative regulator of the methods is a diaryl sulfide and the regulatory binding is reversible.

The invention also provides methods to ameliorate a pathology arising from LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of administering to an individual in need thereof a negative regulator of LFA-1 binding to the ligand in an amount effective to inhibit LFA-1 binding to the ligand, said negative regulator binding to an LFA-1 regulatory site selected from the group consisting of a site that binds a diaryl sulfide, a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide.

The invention also provides LFA-1 α_L polypeptides and fragments thereof comprising a regulatory binding site presenting a diaryl sulfide binding conformation. In one aspect, the LFA-1 polypeptide fragment comprises the α_L polypeptide I domain sequence. Preferably, the LFA-1 polypeptide contains less than all amino acids in the α polypeptide I domain. The invention also provides mutant LFA-1 polypeptides wherein amino acid residues in the wild type α_L polypeptide regulatory site are substituted with non-naturally occurring (*i.e.*, residues not found in the same position in the wild type molecule) amino acid residues. Preferred mutant regulatory sites exhibit modified affinity and/or avidity for an ICAM, both in the presence and absence of an inducing agent (*e.g.*, the monoclonal antibody 240Q described below which induces LFA-1 into an activated state required for ICAM binding). Presently preferred mutants include (i) those demonstrating wild type levels of ICAM-1 binding with or without monoclonal antibody 240Q induction, exemplified mutations having one or more of the single amino acid changes Val^{157}→Ala, Glu^{218}→Ala, Thr^{231}→Ala, Lys^{280}→Ala, and Lys^{294}→Ala, (ii) mutants that support greater than wild type levels of binding without induction and wild type levels

with induction, exemplified by mutations having one or more of the single amino acid changes Ile^{235}-Ala, Ile^{255}-Ala, Ser^{283}-Ala, Glu^{284}-Ala, Glu^{301}-Ala, and Ile^{306}-Ala, (iii) mutants with decreased levels of ICAM-1 binding relative to wild type binding in the absence of induction, but wild-type levels with antibody 240Q induction, exemplified by mutants having one or more of the substitutions Lys^{160}-Ala, Lys^{232}-Ala, Asp^{253}-Ala, Lys^{287}-Ala, Gln^{303}-Ala, Lys^{304}-Ala, and Lys^{305}-Ala, and (iv) mutants demonstrating severely decreased levels or no ICAM-1 binding with or without induction, exemplified by a mutant with the single substitution Tyr^{307}-Ala.

The invention also provides an LFA-1-activating monoclonal antibody secreted by a hybridoma designated 240Q, mailed on March 30, 1999 to, and received on March 31, 1999 by the American Type Culture Collection, 10861 University Blvd., Manassas, VA 20010-2209, and assigned Accession No: HB-12692.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel *in vivo* and *in vitro* methods for negatively, and preferably reversibly, regulating LFA-1 binding to a natural ligand that binds LFA-1 involving use of compounds which bind LFA-1 at a regulatory domain located remote from the ligand binding site. The LFA-1 regulatory site presents a conformation that binds a substituted diaryl sulfide. The binding site is defined by human LFA-1 amino acid residues Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161} and Ile^{306}. Alternatively, the site is defined by amino acid residues Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161} Ile^{306}, Leu^{302}, Tyr^{257}, Leu^{132}, Val^{233}, Val^{130}, and Tyr^{166}. In still another alternative, the binding site is defined by amino acid residues Lys^{287}, Leu^{298}, Ile^{259}, Leu^{302}, Ile^{235}, Val^{157}, Tyr^{257}, Lys^{305}, Leu^{161}, Leu^{132}, Val^{233}, Ile^{255}, Val^{130}, Tyr^{166}, Ile^{306}, Phe^{134}, Phe^{168}, Phe^{153}, Tyr^{307}, Val^{308}, Ile^{309}, Thr^{231}, Glu^{284}, Phe^{285}, Glu^{301}, Met^{154}, Ile^{237}, Ile^{150}, and Leu^{295}. Preferably, the ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

In a presently preferred embodiment, reversible negative regulation (*i.e.*, reversible inhibition) of LFA-1 binding to ligand ICAM is provided by substituted diaryl sulfide compounds which bind LFA-1 at the aforementioned

regulatory domain and/or compounds that competitively inhibit diaryl sulfide binding to said domain.

In one aspect, methods of the invention are carried out using LFA-1 and a binding partner protein, such as ICAM-1, which are recombinant, purified from natural sources, or synthetic. In a preferred method of the invention, the LFA-1 and ICAM binding partner proteins are recombinant. The binding partner proteins may be holoproteins (*e.g.*, including both α and β chains of LFA-1), protein subunits (*e.g.*, the isolated LFA-1 α polypeptide chain), or fragments thereof, including, for example, extracellular domains of either LFA-1 or the ICAM, I domain fragments of LFA-1, less than complete I domain fragments of LFA-1, and/or less than a complete extracellular domain of the ICAM.

In another aspect, the invention provides methods wherein either LFA-1, the ligand, or both are expressed in a cell. When one or both binding partner proteins are expressed in a cell, the cell can be one that expresses an endogenous polynucleotide encoding LFA-1 or the ligand, or a host cell transformed and transfected with a heterologous polynucleotide encoding LFA-1 or the ligand and grown under conditions appropriate to permit expression of LFA-1 or the ligand on the cell surface. Regardless of whether cells of the methods express endogenous or heterologous polynucleotides encoding LFA-1 or the ligand, transcription of the polynucleotide can be directed by either endogenous or heterologous transcriptional control elements. For example, endogenous control elements can be purified from a desired host cell and ligated in an operative position relative to the LFA-1 or the ligand-encoding polynucleotide. Alternatively, a cell expressing endogenous LFA-1 or the ligand can be modified, for example through homologous recombination, to provide the LFA-1 or ligand polynucleotide with one or more transcriptional control elements that modify wild type levels of proteins expression. In assays involving cells expressing endogenous LFA-1 and ligand, preferred cells are leukocytes, *i.e.*, lymphocytes, monocytes, and granulocytes (*e.g.*, neutrophils), and endothelial cells.

In another aspect, the invention embraces methods to inhibit leukocyte adhesion to endothelial cells associated with LFA-1, expressed on leukocytes, binding to an ICAM that binds LFA-1, expressed on endothelial cells. Leukocyte adhesion to

endothelium is characteristic of an inflammatory response arising from release of cell mediators at an injury site. By providing methods to inhibit leukocyte adhesion to endothelial cells, the invention also comprehends methods to inhibit an inflammatory response associated with LFA-1 binding to a natural ligand that binds LFA-1.

Therapeutic Methods

To the extent that leukocyte adhesion to endothelial cells gives rise to a pathological disorder, the invention provides methods to ameliorate pathologies associated with accumulation of leukocytes resulting from LFA-1 binding to an ICAM that binds LFA-1, comprising the step of administering to an individual in need thereof an amount of an inhibitor of LFA-1 binding to the ICAM effective to inhibit LFA-1 binding to the ICAM, said inhibitor binding to LFA-1 at a site presented by amino acid residues Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161} and Ile^{306}. Exemplary medical conditions include, without limitation, inflammatory diseases, autoimmune diseases, reperfusion injury, myocardial infarction, stroke, hemorrhagic shock, organ transplant, and the like. Methods of the invention provide for amelioration of a variety of pathologies, including, for example, but not limited to adult respiratory distress syndrome, multiple organ injury syndrome secondary to septicemia, multiple organ injury secondary to trauma, reperfusion injury of tissue, acute glomerulonephritis, reactive arthritis, dermatosis with acute inflammatory components, stroke, thermal injury, Crohn's disease, necrotizing enterocolitis, granulocyte transfusion associated syndrome, cytokine induced toxicity, and T cell mediated diseases.

Inflammatory cell activation and excessive or unregulated cytokine (e.g., TNFα and IL-1β) production are also implicated in disorders such as rheumatoid arthritis, osteoarthritis, gouty arthritis, spondylitis, thyroid associated ophthalmopathy, Behcet disease, sepsis, septic shock, endotoxic shock, gram negative sepsis, gram positive sepsis, toxic shock syndrome, asthma, chronic bronchitis, allergic respiratory distress syndrome, chronic pulmonary inflammatory disease, such as chronic obstructive pulmonary disease, silicosis, pulmonary sarcoidosis, reperfusion injury of the myocardium, brain, and extremities, fibrosis, cystic fibrosis, keloid formation, scar formation, atherosclerosis, transplant rejection disorders, such as graft vs. host

reaction and allograft rejection, chronic glamerulonephritis, lupus, inflammatory bowel disease, such as ulcerative colitis. proliferative lymphocyte diseases, such as leukemia, and inflammatory dermatoses, such as atopic dermatitis, psoriasis, urticaria, and uveitis.

Other conditions characterized by elevated cytokine levels include brain injury due to moderate trauma (see *J. Neurotrauma*, *12*, pp. 1035-1043 (1995); *J. Clin. Invest.*, *91*, pp. 1421-1428 (1993)), cardiomyopathies, such as congestive heart failure (see *Circulation*, *97*, pp. 1340-1341 (1998)), cachexia, cachexia secondary to infection or malignancy, cachexia secondary to acquired immune deficiency syndrome (AIDS), ARC (AIDS related complex), fever myalgias due to infection, cerebral malaria, osteoporosis and bone resorption diseases, keloid formation, scar tissue formation, and pyrexia.

The ability of the negative regulators of the invention to treat arthritis can be demonstrated in a murine collagen-induced arthritis model [Kakimoto, *et al. Immunol. 142*:326-337 (1992)], in a rat collagen-induced arthritis model [Knoerzer, *et al., Toxical Pathol. 25*:13-19 (1997)], in a rat adjuvant arthritis model [Halloran, *et al., Arthritis Rheum 39*:810-819 (1996)], in a rat streptococcal cell wall-induced arthritis model [Schimmer, *et al., J. Immunol. 160*:1466-1477 (1998)], or in a SCID-mouse human rheumatoid arthritis model [Oppenheimer-Marks, *et al., J. Clin. Invest 101*:1261-1272 (1998)].

The ability of the negative regulators to treat Lyme arthritis can be demonstrated according to the method of Gross, *et al., Science, 218*:703-706, (1998).

The ability of the negative regulators to treat asthma can be demonstrated in a murine allergic asthma model according to the method of Wegner, *et al., Science, 247*:456-459, (1990), or in a murine non-allergic asthma model according to the method of Bloemen, *et al., Am. J. Respir. Crit. Care Med. 153*:521-529 (1996).

The ability of the negative regulators to treat inflammatory lung injury can be demonstrated in a murine oxygen-induced lung injury model according to the method of Wegner, *et al., Lung, 170*:267-279, (1992), in a murine immune complex-induced lung injury model according to the method of Mulligan, *et al., J. Immunol.*,

154:1350-1363, (1995), or in a murine acid-induced lung injury model according to the method of Nagase, *et al.*, *Am. J. Respir. Crit. Care Med.*, *154*:504-510, (1996).

The ability of the negative regulators to treat inflammatory bowel disease can be demonstrated in a murine chemical-induced colitis model according to the method of Bennett, *et al.*, *J. Pharmacol. Exp. Ther.*, *280*:988-1000, (1997).

The ability of the negative regulators to treat autoimmune diabetes can be demonstrated in an NOD mouse model according to the method of Hasagawa, *et al.*, *Int. Immunol. 6*:831-838 (1994), or in a murine streptozotocin-induced diabetes model according to the method of Herrold, *et al.*, *Cell Immunol. 157*:489-500, (1994).

The ability of the negative regulators to treat inflammatory liver injury can be demonstrated in a murine liver injury model according to the method of Tanaka, *et al.*, *J. Immunol.*, *151*:5088-5095, (1993).

The ability of the negative regulators to treat inflammatory glomerular injury can be demonstrated in a rat nephrotoxic serum nephritis model according to the method of Kawasaki, *et al.*, *J. Immunol.*, *150*:1074-1083 (1993).

The ability of the negative regulators to treat radiation-induced enteritis can be demonstrated in a rat abdominal irradiation model according to the method of Panes, *et al.*, *Gastroenterology*, *108*:1761-1769 (1995).

The ability of the negative regulators to treat radiation pneumonitis can be demonstrated in a murine pulmonary irradiation model according to the method of Hallahan, *et al.*, *Proc. Natl. Acad. Sci (USA)*, *94*:6432-6437 (1997).

The ability of the negative regulators to treat reperfusion injury can be demonstrated in the isolated heart according to the method of Tamiya, *et al.*, *Immunopharmacology*, *29*:53-63 (1995), or in the anesthetized dog according to the model of Hartman, *et al.*, *Cardiovasc. Res. 30*:47-54 (1995).

The ability of the negative regulators to treat pulmonary reperfusion injury can be demonstrated in a rat lung allograft reperfusion injury model according to the method of DeMeester, *et al.*, *Transplantation*, *62*:1477-1485 (1996), or in a rabbit pulmonary edema model according to the method of Horgan, *et al.*, *Am. J. Physiol. 261*:H1578-H1584 (1991).

The ability of the negative regulators to treat stroke can be demonstrated in a rabbit cerebral embolism stroke model according to the method of Bowes, *et al.*, *Exp. Neurol.*, *119*:215-219 (1993), in a rat middle cerebral artery ischemia-reperfusion model according to the method of Chopp, *et al.*, *Stroke*, *25*:869-875 (1994), or in a rabbit reversible spinal cord ischemia model according to the method of Clark *et al.*, *Neurosurg.*, *75*:623-627 (1991). The ability of the negative regulators to treat cerebral vasospasm can be demonstrated in a rat experimental vasospasm model according to the method of Oshiro, *et al.*, *Stroke, 28*:2031-2038 (1997).

The ability of the negative regulators to treat peripheral artery occlusion can be demonstrated in a rat skeletal muscle ischemia/reperfusion model according to the method of Gute, *et al.*, *Mol. Cell Biochem.*, *179*:169-187 (1998).

The ability of the negative regulators to treat graft rejection can be demonstrated in a murine cardiac allograft rejection model according to the method of Isobe, *et al.*, *Science*, *255*:1125-1127 (1992), in a murine thyroid gland kidney capsule model according to the method of Talento, *et al.*, *Transplantation*, *55*:418-422 (1993), in a cynomolgus monkey renal allograft model according to the method of Cosimi, *et al.*, *J. Immunol.*, *144*:4604-4612 (1990), in a rat nerve allograft model according to the method of Nakao, *et al.*, *Muscle Nerve*, *18*:93-102 (1995), in a murine skin allograft model according to the method of Gorczynski and Wojcik, *J. Immunol. 152*:2011-2019, (1994), in a murine corneal allograft model according to the method of He, *et al.*, *Opthalmol. Vis. Sci.*, *35*:3218-3225 (1994), or in a xenogeneic pancreatic islet cell transplantation model according to the method of Zeng, *et al.*, *Transplantation*, *58*:681-689 (1994).

The ability of the negative regulators to treat graft-vs.-host disease (GVHD) can be demonstrated in a murine lethal GVHD model according to the method of Harning, *et al.*, *Transplantation*, *52*:842-845 (1991).

The ability of the negative regulators to treat cancers can be demonstrated in a human lymphoma metastasis model (in mice) according to the method of Aoudjit, *et al.*, *J. Immunol.*, *161*:2333-2338, (1998).

Regulatory Binding Site

The invention also provides an LFA-1 regulatory binding site. The regulatory binding site is displayed on the α_L chain of LFA-1 in its wild type, or native, conformation. Fragments of the α_L chain that display the regulatory site are also contemplated, and preferred fragments of the invention include α_L chain I domain sequences, as well as fragments consisting of less than a complete α_L chain I domain. The invention provides LFA-1 regulatory binding sites as part of a polypeptide comprising a human LFA-1 amino acid sequence, the amino acid sequence of a species homolog of human LFA-1, the amino acid sequence of analogs of human LFA-1, or the amino acid sequence of a synthetic polypeptide with homology to human LFA-1. Regulatory binding sites displayed on synthetic polypeptide-like mimetics are also contemplated.

The regulatory binding site of the invention binds a diaryl sulfide (alternatively referred to as a diaryl thioether compound) comprising a first aryl ring and second aryl ring linked to one another through a sulfur atom. In one aspect, the site is defined by human LFA-1 amino acid residues Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161} and Ile^{306}. Alternatively, the binding site is defined by other amino acid residues (*i.e.*, conservative substitutions) or compounds that mimic the binding ability of a site defined by LFA-1 Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161} and Ile^{306}. The regulatory site is also defined by LFA-1 α_L polypeptide amino acid residues that present a domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)-phenyl]sulfide. Preferably, the regulatory site of the invention reversibly binds a negative regulator compound.

The invention also provides LFA-1 regulatory binding site mutants wherein one or more amino acid residues defining the site (*i.e.*, presenting the (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide binding site) is substituted with an alternative amino acid residue, wherein substitution of the wild type amino acid residues results in modified capacity for the mutant to bind (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)-ethenyl)phenyl]sulfide compared to a wild type regulatory site. Preferred mutant regulatory sites exhibit modified affinity and/or avidity for ICAM-1, both in the

presence and absence of an agent that induces ICAM-1 binding (*e.g.*, the monoclonal antibody 240Q which induces LFA-1 into an activated state required for ICAM binding). Presently preferred mutants include (i) those demonstrating wild type levels of ICAM-1 binding with or without monoclonal antibody 240Q induction, exemplified by mutants having one or more of the amino acid changes Val^{157}→Ala, Glu^{218}→Ala, Thr^{231}→Ala, Lys^{280}→Ala, and Lys^{294}→Ala, (ii) mutants that support greater than wild type levels of binding without induction and wild type levels with induction, exemplified by mutants having one or more of the amino acid changes Ile^{235}→Ala, Ile^{255}→Ala, Ser^{283}→Ala, Glu^{284}→Ala, Glu^{301}→Ala, and Ile^{306}→Ala, (iii) mutants with decreased levels of ICAM-1 binding relative to wild type in the absence of induction, but wild-type levels with antibody 240Q induction, exemplified by mutants having one or more of the amino acid substitutions Lys^{160}→Ala, Lys^{232}→Ala, Asp^{253}→Ala, Lys^{287}→Ala, Gln^{303}→Ala, Lys^{304}→Ala, and Lys^{305}→Ala, and (iv) mutants demonstrating severely decreased levels or no ICAM-1 binding with or without induction, exemplified by a mutant with the substitution Tyr^{307}→Ala.

Mutants of the LFA-1 regulatory site are useful in production of antibodies that more precisely define LFA-1 epitopes that can serve as targets for therapeutic intervention. As another example, soluble regulatory sites (or LFA-1 regulatory sites as part of chimeric compounds) with an increased ability to bind an ICAM that binds LFA-1 can modulate LFA-1 binding to the ICAM through competitive inhibition.

Screening Methods

The invention further provides methods for identifying a negative regulator of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 and the ICAM in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ICAM, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ICAM and which binds LFA-1 α_L polypeptide at a site presenting a diaryl sulfide binding conformation defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1. An IC_{50} value for a compound is defined as the concentration of the compound

required to produce 50% inhibition of a biological activity of interest. As used herein, a negative regulator is defined as a compound characterized by an IC_{50} for inhibition of LFA-1 binding to a natural ligand. Negative regulators of LFA-1 binding are defined to have an IC_{50} of less than about 200 μM, less than about 100 μM, less than about 50 μM, and preferably from about 0.05 μM to 40 μM. In another aspect, the invention provides methods for identifying a negative regulator of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 and the ICAM under conditions that allow binding of LFA-1 to the ICAM in the presence and absence of a test compound, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ICAM and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide. Alternatively, the negative regulator competes with 4-amino-2-chlorophenyl-(1'-chloro-2-naphthylphenyl)-sulfide for binding to LFA-1 α_L polypeptide.

In addition, the regulatory site is defined as the site binding site for a negative regulatory that competes for binding to LFA-1 with one of 3-chloro-4-(1-chloro-naphthalen-2-ylsulfanyl)-phenylamine, 2-iso-propylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, (4-methylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, 3-chloro-4-(2-chloro-4-(N,N-dimethylamino)-phenylsulfanyl)-phenylamine, [3-chloro-4-(4-isopropylphenyl-sulfanyl)phenyl]methylamine, (2,4,-dichlorophenyl)[2-chloro-4-(E-((3-(1-pyrrolidin-2-onyl)propylamino)carbonyl)ethenyl)phenyl]sulfide, (2-methylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, (2-formylphenyl)[2-nitro-4-E-((4-acetylpiperazin-1yl)carbonyl)ethenyl)phenyl]sulfide, and 1-[4-(2-isopropyl-phenylsulfanyl)-piperidin-1-yl]ethanone.

The invention also provides methods to identify candidate compounds particularly useful as negative regulators of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-

acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to the LFA-1 α_L polypeptide. The invention therefore provides a method to screen for candidate negative regulators and/or to confirm the mode of action of compounds that decrease LFA-1 binding to an ICAM.

The methods of the invention to identify negative regulators are particularly amenable to the various high throughput screening techniques known in the art. There are a number of different libraries used for the identification of small molecule modulators in these screening techniques of the invention, including, (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" or "leads" via natural product screening. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see *Science 282*:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers, *Curr. Opin. Biotechnol. 8*:701-707 (1997). Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

In high throughput screening methods embraced by the invention, robotic methods are contemplated wherein libraries comprising tens to hundreds of thousands of compounds can be rapidly and efficiently screened.

The invention further provides novel compounds identified as negative regulators in methods of the invention. Negative regulators of the invention are compounds that decrease LFA-1 binding to an ICAM (as compared to binding in the absence of the compound) and compete with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to the α_L polypeptide of LFA-1. Presently preferred inhibitors are substituted diaryl sulfides. Exemplary compounds include those as described in co-pending U.S. patent applications entitled "Cell Adhesion-Inhibiting Antiinflammatory and Immune Suppressive Compounds" filed April 2, 1999, attorney docket number 6446.US.Z3, Serial Number 09/286,645, incorporated herein by reference in its entirety, and "Inhibitors of LFA-1 Binding to ICAMs and Uses Thereof" filed April 2, 1999, attorney docket number 27866/35374, USSN 09/285,325, incorporated herein by reference in its entirety.

The invention also provides compositions comprising negative regulators of the invention, and preferably pharmaceutical compositions further comprising a pharmaceutically acceptable diluent or carrier. Pharmaceutical compositions are particularly useful for treatment of a variety of pathological disorders in humans or other animals, *e.g.*, disorders amenable to animal models as described above.

The invention further provides use of a negative regulator identified by a method of the invention in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to an ICAM that binds LFA-1.

The invention also provides kits to identify inhibitors of LFA-1 binding to an ICAM that binds LFA-1, comprising one or more of a purified and isolated LFA-1 polypeptide, a purified and isolated ICAM polypeptide that binds LFA-1, cells expressing LFA-1, and cells expressing the ICAM. Appropriate control reagents and buffers are contemplated in kits of the invention.

The present invention is illustrated by the following examples. Example 1 describes a high throughput assay to identify inhibitors of LFA-1 binding to full length ICAM-1. Example 2 relates to identification of LFA-1 residues that

participate in antagonist binding. Example 3 describes production of an antibody that activates LFA-1. Example 4 describes identification of an ICAM-1 binding site on LFA-1.

Example 1
High Throughput Screening for LFA-1/ICAM-1 Binding Inhibitors

In an effort to identify inhibitors of LFA-1/ICAM-1 binding, a high throughput screening (HTS) assay was designed to efficiently screen large numbers of chemical compounds in a proprietary library as follows.

Preliminary experiments were carried out in order to define the linear range of LFA-1/ICAM-1 interaction. Recombinant ICAM-1/IgG1 fusion protein (comprising full length ICAM-1) was prepared as described in U.S. Patent Nos. 5,770,686, 5,837,478, and 5,869,262, each of which is incorporated herein by reference. The extracellular domain of ICAM-1 was subcloned into plasmid pDC1 by standard methods to generate an expression construct encoding a chimeric protein containing the ICAM-1 extracellular domain fused to the Fc region of the heavy chain of human IgG1 just upstream of the hinge. The protein was expressed in CHO cells and purified using protein A Sepharose®. The fusion protein was biotinylated using a kit obtained from Pierce Chemical (Rockford, IL). Biotinylated protein (BioIgICAM-1) concentration was determined by measuring absorbance at 280 nm, and serial dilutions were prepared to give a final concentration range of 50 µg/ml to 0.008 µg/ml. Titration of BioIgICAM-1 was carried out with the protein first aliquoted into wells on an assay plate. Recombinant LFA-1 was added to each well at the same concentration and the experiment (as described below) was carried out to completion. The amount of binding was determined for each well, and from a subsequent plot of the results, a single concentration of BioIgICAM-1 was selected for subsequent experiments. In a similar manner, LFA-1 was titrated using the BioIgICAM-1 concentration selected as described above.

On day 1 of the HTS procedure, the capture antibody, *i.e.*, a non-blocking anti-LFA-1 monoclonal antibody (TS2/4.1; ATCC #HB244), was diluted in plate coating buffer (50 mM sodium carbonate/bicarbonate, 0.05% ProClin® 300, pH 9.6) to a final concentration of 2 µg/ml. Immulon® 4 (Dynex

Technologies. Chantilly, VA) plate wells were coated with 100 µl diluted antibody solution per well, and incubation was carried out overnight at 4°C. On day 2, the plates were warmed to room temperature and washed two times with wash buffer (calcium- and magnesium-free phosphate buffered saline, CMF-PBS) with 0.05% Tween®-20). To each well, 200 µl of blocking solution (5% fish skin gelatin in CMF-PBS with 0.05% ProClin® 300) was added, and the blocking incubation was carried out at room temperature for 30 min. The blocking solution was removed by aspiration, and the plates were not washed. LFA-1 was diluted to a final concentration of 1 µg/ml in assay buffer (1% fish skin gelatin and 2 mM $MgCl_2$ in CMF-PBS), and 100 µl was added to each well. Incubation was carried out for one hour, and the plates were washed two times with wash buffer.

A 2X stock solution of BioIgICAM-1 was prepared containing 0.1 µg/ml BioIgICAM-1 and 4 µM crystal violet (an activator of LFA-1/ICAM-1 binding) in Assay Buffer (EG&G Wallac, Gaithersburg, MD). Diluted aliquots (50 µl) of pooled chemicals (22 compounds/pool) from the chemical library were added to the wells, followed by addition of 50 µl of the 2X stock of BioIgICAM-1 to provide a final assay volume of 100 µl (containing 2% DMSO). The plates were incubated for one hour at room temperature and washed once with wash buffer. Europium-labeled streptavidin (Eu-SA; #1244-360, EG&G Wallac) was diluted 1:500 in Assay Buffer, 100 µl of the diluted Eu-SA was added to each well, and the plates were incubated at room temperature for one hour.

Plates were washed eight times with wash buffer, 100 µl of DELFIA® enhancement solution (EG&G Wallac) diluted 1:2, was added to each well, and the plates were shaken for five minutes using a Wallac shaker at fast speed. Plates were read using a Wallac DELFIA® fluorescence reader (fluorimeter). Controls included both positive and negative wells and 50% binding wells established using blocking antibodies, *i.e.*, anti-LFA-1 monoclonal antibody (TS1/22.1, ATCC #HB202) or an anti-ICAM-1 monoclonal antibody. Chemical pools in wells showing 50% or greater inhibition of LFA-1 binding to ICAM-1 were identified and the experiment was repeated using individual chemicals from those pools. Inhibitors of LFA-1/ICAM-1 binding were identified, and a further screen was performed to determine dose

dependence of the inhibitory activity. Further study of selected compounds was carried out using biochemical and cellular assay techniques.

The HTS assay identified more than 40 compounds as hits demonstrating potency in inhibiting LFA-1/ICAM-1 interaction. Of these many, compounds exhibited a diaryl sulfide structure, thereby identifying these compounds as a class of LFA-1/ICAM-1 binding inhibitors.

Example 2
Identification of an LFA-1 Regulatory Binding Site

A. LFA-1 Antagonist Binding to the LFA-1 I domain

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a useful technique to detect small molecule binding to proteins. This technique for screening, or establishing the structure activity relationship (SAR) by NMR [Shuker, *et al., Science 274*:1531-1534 (1996), incorporated herein by reference], has been successful to identify drug leads against several proteins [WO 97/18471, published May 22, 1997 and WO 97/18469, published May 22, 1997, both of which are incorporated herein by reference]. This technique relies on detecting chemical shifts of amide proton and nitrogen atoms resulting from changes in the chemical environment of the peptide backbone, such as those that occur upon ligand binding. Based on the technique's sensitivity, experiments were designed to evaluate binding of small molecule antagonists to LFA-1 in order to understand the structural basis for chemical inhibition of LFA-1 binding to ICAM-1.

Intact LFA-1 is too large to study by NMR spectroscopy. However, evidence indicates that the α_L chain I domain of LFA-1 is largely responsible for ICAM-1 binding, and recombinant I domain polypeptides can compete with intact LFA-1 for ICAM-1 binding. The approximately 200 amino acid I domain region was therefore subcloned, and the recombinant polypeptide was used in NMR experiments to assess whether antagonists of LFA-mediated adhesion interact with the I domain.

The I domain polypeptide corresponding to residues 127-309 in SEQ ID NO: 1 was isotopically labeled in *E. coli* and purified. The pET15b plasmids encoding residues 127-310, 127-309, or 127-305 of SEQ ID NO: 2 were prepared by

PCR amplification of the respective sequences using the human LFA-1 gene as a template and cloned using standard techniques. Each expression plasmid was checked by sequencing.

For NMR experiments, uniformly ^{15}N- or ^{15}N-, ^{13}C-labeled protein was prepared by growing the *E. coli* strain BL21 (DE3) overexpressing the I domain of LFA-1 on M9 medium containing $^{15}NH_4Cl$ with or without [U-^{13}C]-glucose. In addition, [^{15}N, ^{2}H]-labeled proteins, with [^{13}C]-labeled methyl protons in valine and leucine, were prepared to facilitate the interpretation of ^{13}C NOESY experiments [Gardner and Kay, *J. Am. Chem. Sci. 119*:7599 (1997)]. The recombinant I domain was purified using nickel affinity resin according to the manufacturer's suggested protocol. The NMR samples contained 0.8 mM protein, 100 mM sodium phosphate, pH 7.2, in H_2O/D_2O (9:1) or 99.9% D_2O.

All NMR spectra were acquired at 30°C on Bruker DRX500 or DRX600 NMR spectrometers. Backbone resonances were assigned using the HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HNCO and HN(CA)CO triple resonance experiments using uniformly ^{15}N,^{13}C labeled protein. Sidechain assignments were made using the HACACO, HBHA(CO)NH, ^{15}N Edited TOCSY and the HCCH-TOCSY three dimensional experiments. Distance restraints were obtained from ^{13}C-resolved 3D NOESY and ^{13}C edited-filtered NOESY experiments.

Models for the bound compounds were generated with a simulated annealing protocol using the program XPLOR. The docking calculations were performed using the NMR derived distance constraints. The starting protein coordinates in these calculations were derived from the x-ray crystal structure [Qu and Leahy, *Proc. Natl. Acad. Sci.(USA) 92*:10277-10281 (1995)]. Starting structures for the compound were generated randomly. The backbone atoms of the protein were fixed in the docking calculations.

The two dimensional heteronuclear single quantum correlation (HSQC) spectra of the ^{15}N-labeled I domain was indicative of a folded structure. Addition of (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide induced multiple chemical shift changes in the

LFA-1 domain spectrum thereby confirming that the I domain of LFA-1 binds to this antagonist.

B. Binding Interface of Small Molecule Ligand with LFA-1 I domain

To identify the amino acids whose chemical shifts were perturbed by the antagonist, *i.e.*, to map the negative regulator binding site, backbone and side chain resonance assignments of the protein were made using standard heteronuclear NMR experiments. The secondary structure of truncated LFA-1 I-domain protein was compared to that of the x-ray crystal structure of the I domain in intact LFA-1 [Qu and Leahy, *supra*], using both nuclear Overhauser effects (NOE) and backbone chemical shifts. Data indicated that the secondary structure of the I domain in the truncated protein was identical to that found in the previously defined LFA-1 crystal structure. As a result, the antagonist-induced chemical shift changes, as determined by NMR, could then be reliably mapped onto the three-dimensional structure of the I domain, as determined by x-ray crystallography.

In these studies, the largest chemical shift changes occurred for residues that lined a cleft between the carboxy terminal helix of the I domain and central beta sheets. Residues adjacent the metal binding site (MIDAS) showed no shift upon negative regulator binding.

More detailed analysis of (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide binding to LFA-1 was based on NOE experiments. Protons of the protein and negative regulator that were within 5 Å of each other were identified. Resonances that shifted upon negative regulator binding were reassigned by following the shift changes that occurred during a titration of ligand binding and by comparing the pattern of NOEs observed between protons on the protein in the presence and absence of negative regulator. Both ^{13}C edited and ^{13}C edited-filtered NOE experiments were used to identify NOEs between the negative regulator and protein.

Thirty-nine regulatory site contacts were identified and used to dock the negative regulator into the protein. The inter-protein NOEs that were observed in the complex are similar to those predicted by the crystal structure for the I domain.

Based on this observation, the negative regulator/free crystal structure coordinates were used as the starting conformation for the protein in a proposed model of the protein/regulator complex.

Negative regulators were docked using the NOE constraints and the program XPLOR. In the docking calculations, the protein backbone was kept rigid, but amino acid sidechains of the protein were allowed to relax to accommodate the ligand. Only minor changes in protein conformation were necessary to dock the regulator. In all of the docking calculations, the negative regulator was found to lie in the cleft between beta sheet 5 and the carboxy terminal helix, alpha 7 in the I domain, in agreement with the model based on chemical shift changes. The top of the binding pocket is formed from the loop connecting alpha helix 7 to beta sheet 5. Negative regulator ring A is positioned to the top part of the cleft by NOE constraints to Ile^{259} and Leu^{298} while ring B makes contact to the middle of the cleft with NOEs to Ile^{235}, Val^{157}, Leu^{161} and Ile^{306}. The contacts Val^{157} and Leu^{161} on the helix-5 indicate how deep into the protein pocket ring B sits in the complex. Residue Ile^{235} is positioned near the center of the negative regulator and shows large chemical shifts upon regulator binding.

The number of constraints obtained was not sufficient to generate an exhaustively detailed model of the complex. However, the constraints identified unambiguously place the negative regulator binding site in this cleft of the protein. The low number of constraints was due to low sensitivity of NMR signals from residue in the binding pocket that resulted from chemical exchange broadening. Chemical exchange broadening is often indicative of slow motions between different environments. This is generally observed for loose binding of compounds that do not bind in a single conformation. Indeed, some of the constraints observed between the negative regulator and protein were not consistent with a single conformation. For instance, in the docking calculations, two families of possible conformations were found, one pointing toward beta sheet 5 and Ile^{259}, and another with ring A pointing toward alpha helix 7 and residue Leu^{298}.

Results indicated that the protein binding pocket is lined predominantly by hydrophobic Leu/Val/Ile residues. The hydrophobic pocket is,

however, ringed by several hydrophilic groups of lysine and glutamic acid residues. In the ligand-free crystal structure, these hydrophilic groups shield the hydrophobic binding pocket from solvent, possibly by forming salt bridges. In the model for the complex, these hydrophilic side chains move to accommodate the negative regulator.

Example 3

Production of Activating Monoclonal Antibody 240Q

Female BALB/c mice were immunized with purified recombinant α_d/CD18 (described in U.S. Patent 5,837,478, issued November 17, 1998, and incorporated herein by reference). The protein was captured from CHO cell supernatant with a CD18-specific antibody captured on protein A Sepharose® beads. Column material (including beads and capture antibody) was injected with incomplete Freund's adjuvant. Four immunizations over a seven month period were performed before animal #2480 was sacrificed for harvest and fusion of the spleen.

Hybridomas were screened by ELISA for production of IgG by standard protocols. A secondary ELISA screen was performed to identify hybridoma supernatant reactive with either the integrin α or β chain. Briefly, plates were coated in standard buffer with 100 ng/ml of the $F(ab')_2$ fragment of the CD18-specific antibody, 195N. After a blocking step, CHO cells supernatants containing either soluble α_d/CD18 or CD11a/CD18 were added to the wells and capture of integrins was allowed to continue for four hours at 37°C. Hybridoma supernatants were incubated with bound integrin, after which bound mouse IgG was detected with a horseradish peroxidase-conjugated anti-mouse Fc-specific polyclonal antibody. Hybridomas that reacted with both α_d/CD18 and CD11a/CD18 were presumed to recognize either the common β chain or the leucine zipper region of the recombinant molecule. Supernatants were tested by flow cytometry for recognition of native α_d on α_d-transfected JY cells and HL60 cells. Hybridomas that reacted with neither were presumed to be reactive with the leucine zipper peptides.

Thirty five hybridomas were identified as CD18-specific in the secondary assay. A tertiary screen was performed to determine whether the antibodies exhibited any function in an adhesion assay measuring the interaction between peripheral blood lymphocytes (PBL) and ICAM-1. Briefly, PBL were isolated from

heparin-treated whole blood by centrifugation on a Ficoll® gradient. Monocytes and activated lymphocytes were removed by adherence on plastic. Non-adherent cells were treated with hybridoma supernatants or control antibodies for one hour on ice. As a positive control for activation, phorbol myristate acetate (PMA) was used to stimulate a subset of PBL. Cells were washed once and incubated with ICAM-1 immobilized on microtiter plates. After 45 min at 37°C, bound cells were crosslinked for 12 hr using 2.5% (final concentration) glutaraldehyde. Plates were washed in distilled water and stained with 0.5% (final concentration) crystal violet. Following extensive washing with distilled water, destaining was performed using 66% absolute ethanol. Plates were read on a Beckman ELISA reader with a test filter of 570 nm. Six hybridomas were identified that produced an anti-CD18 monoclonal antibody capable of enhancing PBL binding to ICAM-1 at the same level as the PMA control (three- to four-fold over unstimulated cells). The hybridomas were cloned in successive rounds using a modified limiting dilution method. Five clones survived the cloning process and were retested in the PBL assay and with B and T cells. The antibody 240Q was developed further since it appeared to be more effective at cell stimulation.

Specificity of 240Q was assessed by immunoprecipitation experiments. Biotinylated lysates of HL60 cells, positive for expression of all β_2 integrins, were treated with anti-CD18 antibodies 23I11B, 195N, TS1.18 or with 240Q. Antibody/antigen complexes were isolated with an anti-mouse Ig conjugated to protein A Sepharose® matrix. After resolution of protein by SDS-PAGE, biotinylated species were visualized by detection with streptavidin-HRP and developed with a chemiluminescent reagent (Amersham). Antibody 240Q precipitated the identical series of proteins as the known CD18 antibodies. The bands represented known molecular weight proteins for all of the leukointegrin α chains and the CD18 β chain. Extensive immunocytochemical analyses comparing 240Q staining with that of the other anti-CD18 antibodies indicated that 240Q recognized the β chain.

Further evidence that 240Q recognized the β chain (and not a shared epitope on the α chain) was derived from additional immunoprecipitation experiments. It is known that expression of integrins lacking the transmembrane and

cytoplasmic domains results in secretion of large amounts of the free β chain. While the anti-CD18 antibody 195N will bind to and precipitate free β chain, the 23I11B antibody will recognize β chain only in the context of a heterodimer. Immunoprecipitation of soluble α_d/CD18LZ (leucine zipper) protein from CHO supernatants yields protein that, on SDS-PAGE, is predominantly β chain, with non-stoichiometric amounts of the appropriate alpha chain. In these experiments, the affinity resin is not washed, so disruption of the bound heterodimer would not be expected to affect results.

Several integrin-specific monoclonal antibodies were biotinylated and used in flow cytometry designed to map the 240Q binding site on a coarse level. Cells were incubated with a biotinylated antibody and a different, unlabeled antibody at the same time, and it was determined whether the unlabeled antibody can compete with the labeled antibody. The untreated control consisted of cells stained with the biotinylated antibody alone.

A preliminary experiment was performed to titrate single antibodies with HUT78 (CD11a/CD18$^+$ T cell line) and HL60 (CD11a$^+$, CD11b$^+$, CD11c$^+$ myeloid lineage cell line). Biotinylated antibodies were incubated with both cell types at 0.3, 1.0, 3.0, and 10 μg/ml. Biotinylated antibody was detected with both streptavidin-FITC (to determine whether biotinylation was successful) and anti-mouse Ig/FITC (to determine whether biotinylated antibodies were still functional and binding at equivalent levels). Staining with 240Q with the streptavidin-FITC detection method was only 25% that of the CD18-specific antibody 23I11B at any given concentration. The difference was more dramatic with the anti-mouse FITC detection. Affinity differences would not be expected to account for these results, since transformants stained equally well with both antibodies.

This result implied that 240Q recognizes a specific subset of CD18 molecules on the cell surface, a finding that correlated with previous staining of COS transfectants. Another possibility is that the antibody recognized a particular molecular conformation achieved temporally at only 25% of the time.

In the cross-competition experiments, there appeared to be no overlap between 240Q and any other integrin α or β chain-specific antibodies. This finding

was confirmed by ELISAs with captured recombinant LFA-1 and Mac-1 protein. In these assays, the unlabeled antibody was used to capture the protein and the labeled antibody was used to detect it. Failure of the labeled antibody to bind would indicate that the binding site was occupied by the capture antibody. While 240Q capture blocked binding of biotinylated 240Q, it did not block binding with any other antibody. Capture by CD11a, CD11b, or other CD18 antibodies did not prevent detection by biotinylated 240Q. There was no difference between the ability of 240Q and other CD18-specific antibodies to recognize recombinant CD18 integrins. Treatment of immobilized recombinant LFA-1 with either 240Q or manganese did not enhance ICAM-1 binding, implying that the recombinant integrin was in a constitutively activated conformation.

Based on the observation that 240Q treatment of cells in the cross-blocking experiments caused aggregation, an aggregation assay was run with JY, Jurkat, and HL60 cells. Cells were plated in culture medium and treated with concentrations of 240Q or 195N ranging from 0.2 to 10 μg/ml. After a 30 minute incubation at 37°C, wells were photographed. Antibody 240Q at concentrations from 0.5 - 10 μg/ml appeared to induce substantial aggregation. Antibody 195N did not induce the aggregate phenotype. It was not apparent whether this behavior was due to integrin-CAM interactions or an indirect induction of other adhesive pathways.

The integrin-activation function of 240Q was further characterized in binding experiments using the TACO cell line. These cells were isolated from a patient diagnosed with a subtype of leukocyte adhesion deficiency (LAD). The subtype is characterized by normal surface expression of LFA-1 on lymphocytes, but the inability of LFA-1 to bind ICAM-1. The functional phenotype is not recognized by phorbol myristate acetate (PMA). Treatment of the cells with the antibody 240Q rescued homotypic aggregation, which was determined to be ICAM-1-dependent using an ICAM-1-specific antibody. When subsaturating amounts of the antibody $F(ab')_2$ fragment were used to treat the cells, aggregation did not occur and the 240Q-treated cells were capable of recognizing ICAM-1/Fc protein immobilized on microtiter plates. Cells which were treated with the anti-CD18 antibody and either no

240Q antibody or PMA did not bind immobilized ICAM-1/Fc. This data indicated that the mechanism of integrin activation by PMA and 240Q is distinct.

Example 4
ICAM-1 Binding Site

A. Production and Purification of Recombinant Human ICAM-1 Domains 1 and 2

Domains 1 and 2 of human ICAM-1 were amplified by PCR by standard methods using primers d1/HindIII and d2/XbaI and an ICAM-1 cDNA as template.

d1/HindIII SEQ ID NO: 3

CCCAAGCTTCCGCCGCCACCATGGCTCCCAGCAG

d2/XbaI SEQ ID NO: 4

TGCTCTAGACTGGTGATGGTGATGGT-
GATGAAAGGTCTGGAGCTGGTAGGGG

The amplification product was digested with *Hin*dIII and *Xba*I and gel purified. The purified fragment was used in a three-way ligation including ICAM-1 domains 1 and 2 (the *Hin*dIII/*Xba*I fragment), pDEF17 previously digested with *Xba*I and *Not*I, and pDEF17 previously digested with *Not*I and *Hin*dIII, and the resulting plasmid, pDEF17/ICAM-1 domains 1 and 2, was sequenced. For expression, the plasmid was transformed into CHO cells by standard methods.

A 70 ml immunoaffinity column was created by coupling 2 mg of a non-blocking anti-ICAM-1 18E3D monoclonal antibody per ml of activated CNBr-Sepharose® according to the manufacturer's suggested protocol. The column was equilibrated with 20 mM Tris/150 mM NaCl at pH 7.5. Approximately 2.5 liters of culture supernatant from CHO cells secreting recombinant human ICAM-1 domains 1 and 2 was applied to the column overnight at 4°C. The following morning, the column was washed to baseline protein elution with equilibration buffer. The column was eluted with 2 M KSCN, pH 8.0, and fractions were analyzed by SDS-PAGE under reducing conditions. Samples containing pure ICAM-1 domains 1 and 2 were pooled, the buffer was exchanged into 20 mM Tris/150 mM NaCl/pH 7.5,

and the protein was concentrated ten-fold. Concentration of the protein was determined by absorption at 280 nm using an extinction coefficient of 1.0 AU/1.4 mg of ICAM-1 domain 1 and 2. The purification process and analysis was repeated using the same column and an additional 2.5 liters of CHO culture supernatant. The two pools were combined and filtered.

B. ICAM-1 Binding Interface on the LFA-1 I-Domain

Residues that are important for ICAM-1 binding to the LFA-1 I domain have previously been identified using mutational studies and residues that form the MIDAS region of the I domain have been shown to be important for binding by this approach. Other LFA-1 regions have been investigated using chimeric proteins comprising human and mouse I domains [Huang and Springer, *J. Biol. Chem.* *270*:19008-19016 (1995)]. Because many of the residues in the LFA-1 ligand binding site important for ICAM-1 binding are either identical (Tyr^{307}, Lys^{301}, Lys^{287}) or highly conserved (human Lys^{305}, Lys^{304} - mouse Arg^{305}, Arg^{304}) between mouse and human, chimeric protein studies were unable to specifically identify necessary binding residues. Chemical shift changes that occur upon binding provides a sensitive way to map binding sites. ^{1}H-^{13}C HSQC and ^{1}H-^{15}N HSQC spectra of the I domains of LFA-1 in the presence and absence of ICAM-1 were used to identify residues affected by ICAM-1 domains 1 and 2 fragment binding using NMR techniques as generally described above.

The complex was found to be in slow exchange on the NMR timescale, indicating binding much tighter than 10 μM. Many residues whose NMR signals show the largest changes upon binding were found on the surface of the I domain. In addition, residues near the MIDAS motif and alpha helix 7 of the small molecule ligand binding site were most affected by ICAM-1 binding. These data indicate that the MIDAS motif and alpha helix 7 participate in ICAM-1 binding, either directly by binding the ligand, or indirectly by mediating a conformational change in the I domain. Furthermore, the involvement of the α helix 7 in ICAM-1 binding provides a rationale for how small molecules that bind to this region of the I domain disrupt LFA-mediated adhesion.

C. Functionally Important Residues in the Ligand Binding Pocket

In an attempt to identify functional I domain residues in and around the site of compound binding, amino acid substitution mutants were generated and tested for the ability to bind ICAM-1. Amino acids most affected in NMR by compound binding and whose side chains are directed toward the surface of the I domain were substituted with alanine. In addition, Asp^{137}, a residue located within and essential to a functional MIDAS and ICAM-1 binding site, was substituted with alanine. The various I domain mutants were expressed in COS cells and cell adhesion to ICAM-1 was determined in the presence of a CD18 monoclonal antibody, 240Q, that induces high avidity binding.

1. Generation of the mutations in the CD11a I domain:

Twenty-five individual mutations in the LFA-1 α polypeptide (CD11a) were generated. Each mutation was prepared using Stratagene's QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, two primers were synthesized that introduced a specific mutation in the amplification product. Primers utilized are set out below, with only the sense primer shown.

D137A/S: SEQ ID NO: 5

CTGGTATTTCTGTTTGCGGGTTCGATGAGCTTG

V157A/S: SEQ ID NO: 6

GACTTCATGAAGGATGCGATGAAGAAACTCAGC

K160A/S: SEQ ID NO: 7

GAAGGATGTGATGAAGGCGCTCAGCAACACTTGC

E218A/S: SEQ ID NO: 8

CAATTATGTCGCGACAGCGGTGTTCCGGGAGGAG

T231A/S: SEQ ID NO: 9

GCCCGGCCAGATGCCGCGAAAGTGCTTATCATC

K232A/S: SEQ ID NO: 10

CGGCCAGATGCCACCGCGGTGCTTATCATCATC

I235A/S: SEQ ID NO: 11

GCCACCAAAGTGCTTGCGATCATCACGGATGGG

D253A/S: SEQ ID NO: 12

CATCGATGCGGCCAAAGCGATCATCCGCTACATC

I255A/S: SEQ ID NO: 13

GCGGCCAAAGACATCGCGCGCTACATCATCGGG

K280A/S: SEQ ID NO: 14

CACAAATTTGCATCAGCGCCCGCGAGCGAGTTTG

S283A/S: SEQ ID NO: 15

GCATCAAAACCCGCGGCGGAGTTTGTGAAAATTC

E284A/S: SEQ ID NO: 16

CAAAACCCGCGAGCGCGTTTGTGAAAATTCTG

K287A/S: SEQ ID NO: 17

GCGAGCGAGTTTGTGGCGATTCTGGACACATTTG

K294A/S: SEQ ID NO: 18

CTGGACACATTTGAGGCGCTGAAAGATCTATTC

E301A/S: SEQ ID NO: 19

GAAAGATCTATTCACTGCGCTGCAGAAGAAGATC

Q303A/S: SEQ ID NO: 20

CTATTCACTGAGCTGGCGAAGAAGATCTATGTC

K304A/S: SEQ ID NO: 21

TTCACTGAGCTGCAGGCGAAGATCTATGTCATTG

K305A/S: SEQ ID NO: 22

CACTGAGCTGCAGAAGGCGATCTATGTCATTGAG

I306A/S: SEQ ID NO: 23

GAGCTGCAGAAGAAGGCGTATGTCATTGAGGGC

Y307A/S: SEQ ID NO: 24

CTGCAGAAGAAGATCGCGGTCATTGAGGGCACA

Control mutants included the following, wherein amino acid changes were introduced in regions reported by others to be involved in ICAM-1 binding.

T243A/S: SEQ ID NO: 25

ACGGATGGGGAGGCCGCGGACAGTGGCAACATC

S245A/S: SEQ ID NO: 26

GGGGAGGCCACTGACGCGGGAAACATCGATGC

N247A/S: SEQ ID NO: 27

GCCACTGACAGTGGCGCGATCGATGCGGCCAAAG

D249A/S: SEQ ID NO: 28

GACAGTGGCAACATCGCGGCGGCCAAAGACATC

K252A/S: SEQ ID NO: 29

CAACATCGATGCGGCCGCGGACATCATCCGCTAC

The primers were used in two PCR reactions, one with full-length CD11a (residues 1-1170) in plasmid pDC1 as template and the other with CD11a I domain (residues 152-334) in plasmid pET15b as template. PCR reaction conditions varied depending on the melting temperature (T_M) of the primers. Details of the reaction for each mutation are described below. The general format was as follows: one cycle at 95°C for 30 seconds followed by 16 cycles of: 95°C for 30 seconds, 55°C for one minute, and 68°C for 20 minutes. After PCR, template DNA was digested with *Dpn*I at 37°C for one hour and the remaining amplified DNA was used to transform supercompetent *E. coli* XL1 Blue (Stratagene) according to the manufacturer's suggested protocol. Selected colonies were grown in liquid culture and plasmid DNA was isolated and sequenced. For the full-length mutants, a 1.8 kb *Hin*dIII/*Eco*RI fragment containing the 5' half of the gene was isolated and subcloned into the parental vector. Subclones were sequenced to verify the integrity of the junctions and the presence of the mutation.

2. PCR Conditions

Mutations V157A, E218A, T231A, I235A, I255A, E284A, K287A, K294A, K305A were generated in PCR including a 45°C annealing step and a 58°C extension step. In generating these mutations, extension times for the full length sequence in pDC1 was 20 minutes and 15 minutes for I domain in pET15b.

For mutations D137A, K160A, K232A, K280A, S283A, E301A, Q303A, K304A and I306A, the same temperatures as described above were used, but with both templates, the extension time for both templates was 20 minutes. For mutants Y307A and D253A, an extension step of 25 minutes was used.

For mutants T243A, S245A, N247A, D249A and K252A, the annealing step was carried out at 45°C, and extension was carried out at 60°C for 20 minutes. For mutant S245A, PCR included 18 cycles rather than 16 cycles.

3. COS Cell Transfections

On day 1, COS cells were seeded at 1.6×10^6 cells per 10 cm plate in DMEM 10% FBS (growth media). After 18 to 24 hr, cells were transfected as follows. Seven μg each of CD18/pDC1 and CD11a/pDC1 plasmid DNA was added to three ml OPTI MEM media and 49 μl Lipofectamine was added to another three ml of the same media. The two resulting solutions were mixed, inverted five times, incubated at room temperature for 30 min, and diluted by addition of 6.1 ml OPTI MEM. Cells were washed once with OPTI MEM and the DNA/Lipofectamine mixture was added. Cells in the mixture were incubated at 37°C in CO_2 for six hours. Media containing the plasmid DNA was removed and replaced with growth media. Cells were grown overnight and media was removed and replaced. After overnight growth, cells were split 1:2 and grown overnight again. Cells were removed from the plate with Versene, collected by centrifugation, resuspended in adhesion buffer containing (RPMI containing 5% inactivated FBS), and counted. Cells were then used for adhesion assays and for fluorescence activated cell sorting (FACS) staining and analysis.

4. Adhesion Assay

Adhesion assays were performed in 96-well Easy Wash plates (Corning, Corning NY) using a modification of a previously reported procedure [Sadhu, *et al., Cell. Adhes. Commun. 2*:429-440 (1994)]. Each well was coated overnight at 4°C with (i) 50 μl of ICAM-1/Fc (5 μg/ml), (ii) anti-CD18 monoclonal antibody TS1/18 [Sanchez-Madrid, *et al., Proc. Natl. Acad. Sci. (USA) 79*:7489-7493 (1982); Weber, *et al., J. Immunol. 159*:3968-3975 (1997); Lu, *et al., J. Immunol.*

159:268-278 (1997)] at 5 μg/ml together with anti-CD11a monoclonal antibody TS1/22 at 5 μg/ml in 50 mM bicarbonate buffer, pH 9.6, or (iii) buffer alone. Plates were washed twice with 200 μl per well D-PBS and blocked with 1% BSA (100 μl/well) in D-PBS for one hour at room temperature. Wells were rinsed once with 100 μl adhesion buffer (described above) and 100 μl adhesion buffer was then added to each well. Adhesion buffer (100 μl) with or without blocking antibody TS1/22 at 20 μg/ml was added to each well. COS transfectants (100 μl, approximately 0.75 x 10^6 cells/ml) expressing the heterodimer (with or without a mutation) in adhesion buffer, with or without activating antibody 240Q (10 μg/ml) was added to each well and the plates incubated at 37°C for 15 to 20 min. Adherent cells were fixed by addition of 50 μl/well 14% glutaraldehyde in D-PBS and incubated at room temperature for 1.5 hr. The plates were washed with dH_2O and stained with 100 μl/well 0.5% crystal violet in 10% ethanol for five minutes at room temperature. Plates were washed in several changes of dH_2O. After washing, 70% ethanol was added, and adherent cells were quantitated by determining absorbance at 570 nm and 410 nm using a SPECTRAmax® 250 microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA). Percentage of cells binding was calculated using the formula:

$$\% \text{ cells binding} = \frac{A_{570}-A_{410}(\text{binding to ICAM-1})}{A_{570}-A_{410}(\text{binding to CD18 + CD11a antibody})} \times 100$$

Data were normalized using the formula:

$$\% \text{ wildtype binding} = \frac{(\% \text{ mutant cell binding})}{(\% \text{ wildtype cells binding})} \times 100$$

5. FACS Staining

FACs staining was carried out in a 96 well plate. Each transfectant was stained with an antibody to CD18 (TS1/18), an antibody to CD11a (TS1/22), and an activating antibody to CD18 (240Q). Controls included unstained cells, cells

stained with secondary antibody only, and cells stained with an isotype matched control antibody (1B7).

Briefly, approximately 1 x 10^5 to 5 x 10^5 cells were aliquoted per well and one antibody per well was added per transfectant. Cells were centrifuged in a table top centrifuge at 1200 x *g* for five minutes at 4°C, rinsed in staining buffer (containing ice-cold CMF-PBS 2% FBS), and centrifuged again.

Primary antibody (100 µl at 10 µg/ml) or staining buffer, was added to each well and incubation carried out on ice for 30 min. Cells were pelleted by centrifugation and washed once with staining buffer. Secondary antibody (100 µl), typically sheep anti-mouse Ig-FITC (Sigma), at a 1:200 dilution was added to each well and incubation carried out on ice in the dark for 30 minutes. Cells were pelleted by centrifugation, washed three times with CMF-PBS, and resuspended and fixed in 300 µl 1% formaldehyde. Samples were analyzed on the same day stained.

Results indicated that the mutants could be separated into four phenotypes: 1) mutants that demonstrated wild type levels of binding with or without 240Q induction (Val^{157}→Ala, Glu^{218}→Ala, Thr^{231}→Ala, Lys^{280}→Ala, and Lys^{294}→Ala), 2) mutants that supported greater than wild type levels of binding without 240Q induction and wild type levels with induction (Ile^{235}→Ala, Ile^{255}→Ala, Ser^{283}→Ala, Glu^{284}→Ala, Glu^{301}→Ala, and Ile^{306}→Ala), 3) mutants that possessed decreased levels of binding relative to wild type binding in the absence of induction, but wild-type levels with 240Q induction (Lys^{160}→Ala, Lys^{232}→Ala, Asp^{253}→Ala, Lys^{287}→Ala, Gln^{303}→Ala, Lys^{304}→Ala, and Lys^{305}→Ala), and 4) mutants that demonstrate severely decreased levels or no binding with or without 240Q (Tyr^{307}→Ala).

The effects of mutations on ICAM-1 binding were not due to varying levels of LFA-1 expression, and both CD11a and CD18 were expressed at equivalent levels to that of wild type. For mutants showing significantly decreased binding, ^{15}N labeled I domain was prepared and ^{1}H-^{15}N HSQC spectra were compared to that of wild type I domain. All of these mutant protein spectra were very similar to that of the wild type protein indicating that no significant conformational changes in the protein arose from any of the mutations. Data for the eighth mutant, K304A, could

not be obtained due to poor expression of the protein in bacteria. These mutants all bound 240Q at equal levels.

The analysis indicates that amino acids in and around the site of antagonist binding contribute to a regulatory site for LFA-1 mediated cell adhesion. The residues Lys^{232}, Lys^{287}, Lys^{304}, Lys^{305}, and Tyr^{307} are all hydrophilic residues that surround, but do not directly form, the small molecule ligand binding site. Residues Val^{157}, Ile^{235}, Ile^{255}, and Ile^{306} form the hydrophobic pocket of the small molecule binding site.

6. **Mechanism of Regulation**

LFA-1 binding activity is regulated through two different mechanisms which are not mutually exclusive: 1) control of individual receptor affinity (the strength of binding between two molecules), and 2) control of overall avidity (the affinity multiplied by the number of interactions which are occurring at one time) by the regulated aggregation of individual receptors through interactions with the cellular cytoskeleton. If the LFA-1 regulatory binding site, as defined above, is responsible for regulating individual receptor affinity, then the activating mutants, typified by I235A (described above), should possess higher binding affinity in cellular adhesion. These methods, however, are imprecise and do not accurately separate affinity from avidity. Therefore, in order to accurately measure the relative binding affinity of wildtype and mutant I235A for ICAM-1, the following assay was carried out. Recombinant I235A was produced in CHO cells in secreted form using the same method as that used for production of recombinant LFA-1 in Example 1. The soluble forms of recombinant LFA-1 (used here and in Example 1) and I235A (used here) contain deletions of the transmembrane and cytoplasmic domains of CD11a and CD18 (SEQ ID NO: 30 [full length polynucleotide] and 31 [full length amino acid], and substitution of these regions for acidic and basic leucine zipper cassettes, respectively, which promote and stabilize specific heterodimerization as described for the production of soluble T-cell receptor [Hsiu-Ching *et al Proc. Natl. Acad. Sci. (USA) 91*: 11408-11412 (1994)].

Both wildtype and mutant I235A CD11a were truncated after position Q1063 in the mature polypeptide, and the 47 amino acid acidic leucine zipper cassette (SEQ ID NO: 32) was added in-frame, using standard methods. CD18 was truncated after position N678 in the mature polypeptide, and the 47 amino acid basic leucine zipper cassette (SEQ ID NO: 33) was added in-frame.

Acidic leucine zipper cassette SEQ ID NO: 32

Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro Ser Ala Gln Leu Glu Lys Glu Leu Gln Ala Leu Glu Lys Glu Asn Ala Gln Leu Glu Trp Glu Leu Gln Ala Leu Glu Lys Glu Leu Ala Gln

Basic leucine zipper cassette SEQ ID NO: 33

Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro Ser Ala Gln Leu Lys Lys Lys Leu Gln Ala Leu Lys Lys Lys Asn Ala Gln Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala Gln

Both recombinant LFA-1 and I235A were expressed in CHO cells and purified from the supernatants using separate 8 ml immunoaffinity columns created by coupling 2 mg of anti-CD18 23I11B monoclonal antibody per ml of activated CNBr-Sepharose™ according to the manufacturer's suggested protocol, and eluted using a 20 mM Tris (pH 7.5), 2.5 M $MgCl_2$ buffer. Recombinant LFA-1 and I235A were then purified a second time by gel filtration chromatography over a Pharmacia HiLD SuperDex 200™ column in PBS buffer using standard methods, in order to remove any single chain, aggregated and/or degraded material. The resulting suspensions of purified heterodimers were concentrated using Millipore Ultrafree-4 Centrifugal Filter Units™ with Biomax-30™ membranes, then dialyzed in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM $MgCl_2$) at 4 oC, and quantitated using a BioRad Protein Assay™ and the manufacturer's protocol.

The affinity of recombinant LFA-1 and I235A was then measured by surface plasmon resonance using a BIAcore 2000 biosensor™ (Pharmacia Biosensor AB). All experiments were performed at 25°C. All proteins for injection were diluted in HBS buffer. Anti-human Fc antibody (Pierce) was coupled to a CM5™ sensor chip (Pharmacia Biosensor AB) using an amine-coupling kit (Pharmacia

Biosensor AB). The antibody was injected at 50 mg/ml in 10 mM Na acetate (pH 4.5) buffer until approximately 12,000 RU was bound. For each assay, recombinant ICAM-1/IgG1 (see above) was injected at 10 mg/ml until 200 RU was captured onto the chip through binding to the anti-human Fc antibody. LFA-1 or I235A was then injected at different concentrations, using a flow rate of 10 ml/min, and the surface plasmon resonance was recorded. After each concentration of LFA-1 or I235A was allowed to bind and dissociate, the chip was stripped of ICAM-1/LFA-1 complexes with 0.1N HCl and regenerated with fresh ICAM-1/IgG1 before the next concentration of LFA-1 or I235A was analyzed. The association and dissociation rate constants (k_a and k_d, respectively) for LFA-1 and I235A were calculated using the BIA evaluation 2.0 program and its 1:1 Langmuir binding kinetics model (Pharmacia Biosensor AB). The k_a for LFA-1 and I235A were identical and equaled 2.2 x 105 M^{-1} s^{-1}. However, the k_d for LFA-1 and I235A were significantly different and equaled 1.2 x 10^{-2} s^{-1} and 1.9 x 10^{-3} s^{-1}, respectively. These values corresponded to an affinity dissociation rate (K_D) of 547 nM for LFA-1, which is in close agreement with the value of 500 nM calculated by Tominaga using a similar method [Tominaga, *et al J. Immunol., 161*: 4016-4022 (1998)]. However, the corresponding K_D of 86 nM for I235A, represents a 6-fold increase in affinity for I235A over LFA-1, which was similar to the increase observed in cell-binding assays using COS-7 cell transfectants (discussed above). These data strongly suggest that the activation of LFA-1 binding to ICAM-1 caused by the I235A mutation in the LFA-1 regulatory binding site was a result of an increase in LFA-1 affinity. Therefore, the molecular mechanism whereby the LFA-1 regulatory binding site mediates its effects on LFA-1 binding to ICAM, must be effected in large part through regulation of the affinity state of LFA-1. Based on these data, the diaryl sulfide compounds which bind to the LFA-1 regulatory binding site are predicted to inhibit adhesion to ICAM-1 by lowering the affinity of LFA-1 for ICAM through an increase in the k_d of the receptor or through stabilizing the low affinity state of LFA-1.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those

skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

WHAT IS CLAIMED IS:

1. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site presenting a conformation that binds a diaryl sulfide or a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1.

2. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site that binds a diaryl sulfide or a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, Ile^{306}, Leu^{302}, Tyr^{257}, Leu^{132}, Val^{233}, Val^{130}, and Tyr^{166} of human LFA-1.

3. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site that binds a diaryl sulfide or a site defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, Ile^{306}, Lys^{287}, Leu^{302}, Ile^{257}, Lys^{305}, Leu^{161}, Leu^{132}, Val^{233}, Ile^{255}, Val^{130}, Tyr^{166}, Phe^{134}, Phe^{168}, Phe^{153}, Tyr^{307}, Val^{308}, Ile^{309}, Thr^{231}, Glu^{284}, Phe^{285}, Glu^{301}, Met^{154}, Ile^{237}, Ile^{150}, and Leu^{295}. of human LFA-1.

4. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding under conditions that allow binding of LFA-1 to the ligand in the presence and absence of a test compound, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide.

5. A screening method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) contacting LFA-1 with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide.

6. The method according to any one of claim 1 through 5 wherein the natural ligand is an ICAM.

7. The method according to claim 6 wherein the ICAM is ICAM-1 or ICAM-3.

8. The method of claim 1, 2, 3, 4, 5 or 7 wherein the negative regulator is a diaryl sulfide.

9. A pharmaceutical composition comprising a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 identified by the method of claim 1, 2, 3, 4, or 5.

10. Use of a negative regulator identified by the method of claim 8 in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to a natural ligand that binds LFA-1.

11. A method for inhibiting LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of contacting LFA-1 with a negative regulator compound; said negative regulator binding LFA-1 α_L polypeptide at a site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 α_L polypeptide and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide .

12. A method to inhibit leukocyte adhesion to endothelial cells comprising the step of contacting said leukocyte with a negative regulator of LFA-1 binding to an ICAM that binds LFA-1, said negative regulator binding an LFA-1 regulatory site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 α_L polypeptide and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide.

13. A method to ameliorate a pathology arising from LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of administering to an individual in need thereof a negative regulator of LFA-1 binding to the ligand in an amount effective to inhibit LFA-1 binding to the ligand, said negative regulator binding to an LFA-1 regulatory site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile^{259}, Leu^{298}, Ile^{235}, Val^{157}, Leu^{161}, and Ile^{306} of human LFA-1 and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide.

14. The method of claim 9, 10, 11, 12, or 13 wherein the inhibitor is a diaryl sulfide.

15. The method of claim 11 or 13 wherein the natural ligand is an ICAM.

16. The method of claim 15 wherein the ICAM is ICAM-1 or ICAM-3.

17. A mutant LFA-1 α_L polypeptide comprising an amino acid substitution selected from the group consisting of Val^{157}→Ala, Glu^{218}→Ala, Thr^{231}→Ala, Lys^{280}→Ala, Lys^{294}→Ala, Ile^{235}→Ala, Ile^{255}→Ala, Ser^{283}→Ala, Glu^{284}→Ala, Glu^{301}→Ala, Ile^{306}→Ala, Lys^{160}→Ala, Lys^{232}→Ala, Asp^{253}→Ala, Lys^{287}→Ala, Gln^{303}→Ala, Lys^{304}→Ala, Lys^{305}→Ala, and Tyr^{307}→Ala of SEQ ID NO: 2.

18. A monoclonal antibody secreted by hybridoma 240Q.

SEQUENCE LISTING

<110> Staunton, Donald
Huth, Jeff

<120> LFA-1 Regulatory Binding Site and Uses Thereof

<130> 27866/36400

<140>
<141>

<150> 09/285,477
<151> 1999-04-02

<160> 33

<170> PatentIn Ver. 2.0

<210> 1
<211> 5133
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (164)..(3598)

<400> 1
cctctttcac cctgtctagg ttgccagcaa atcccacggg cctcctgacg ctgcccctgg 60

ggccacaggt ccctcgagtg ctggaaggat gaaggattcc tgcatcactg tgatggccat 120

ggcgctgctg tctgggttct ttttcttcgc gccggcctcg agc tac aac ctg gac 175
Tyr Asn Leu Asp
1

gtg cgg ggc gcg cgg agc ttc tcc cca ccg cgc gcc ggg agg cac ttt 223
Val Arg Gly Ala Arg Ser Phe Ser Pro Pro Arg Ala Gly Arg His Phe
5 10 15 20

gga tac cgc gtc ctg cag gtc gga aac ggg gtc atc gtg gga gct cca 271
Gly Tyr Arg Val Leu Gln Val Gly Asn Gly Val Ile Val Gly Ala Pro
25 30 35

ggg gag ggg aac agc aca gga agc ctc tat cag tgc cag tcg ggc aca 319
Gly Glu Gly Asn Ser Thr Gly Ser Leu Tyr Gln Cys Gln Ser Gly Thr
40 45 50

gga cac tgc ctg cca gtc acc ctg aga ggt tcc aac tat acc tcc aag 367
Gly His Cys Leu Pro Val Thr Leu Arg Gly Ser Asn Tyr Thr Ser Lys
55 60 65

tac ttg gga atg acc ttg gca aca gac ccc aca gat gga agc att ttg 415
Tyr Leu Gly Met Thr Leu Ala Thr Asp Pro Thr Asp Gly Ser Ile Leu
70 75 80

gcc tgt gac cct ggg ctg tct cga acg tgt gac cag aac acc tat ctg 463
Ala Cys Asp Pro Gly Leu Ser Arg Thr Cys Asp Gln Asn Thr Tyr Leu
85 90 95 100

```
agt ggc ctg tgt tac ctc ttc cgc cag aat ctg cag ggt ccc atg ctg   511
Ser Gly Leu Cys Tyr Leu Phe Arg Gln Asn Leu Gln Gly Pro Met Leu
                105                 110                 115

cag ggg cgc cct ggt ttt cag gaa tgt atc aag ggc aac gta gac ctg   559
Gln Gly Arg Pro Gly Phe Gln Glu Cys Ile Lys Gly Asn Val Asp Leu
            120                 125                 130

gta ttt ctg ttt gat ggt tcg atg agc ttg cag cca gat gaa ttt cag   607
Val Phe Leu Phe Asp Gly Ser Met Ser Leu Gln Pro Asp Glu Phe Gln
        135                 140                 145

aaa att ctg gac ttc atg aag gat gtg atg aag aaa ctc agc aac act   655
Lys Ile Leu Asp Phe Met Lys Asp Val Met Lys Lys Leu Ser Asn Thr
    150                 155                 160

tcg tac cag ttt gct gct gtt cag ttt tcc aca agc tac aaa aca gaa   703
Ser Tyr Gln Phe Ala Ala Val Gln Phe Ser Thr Ser Tyr Lys Thr Glu
165                 170                 175                 180

ttt gat ttc tca gat tat gtt aaa tgg aag gac cct gat gct ctg ctg   751
Phe Asp Phe Ser Asp Tyr Val Lys Trp Lys Asp Pro Asp Ala Leu Leu
                185                 190                 195

aag cat gta aag cac atg ttg ctg ttg acc aat acc ttt ggt gcc atc   799
Lys His Val Lys His Met Leu Leu Leu Thr Asn Thr Phe Gly Ala Ile
            200                 205                 210

aat tat gtc gcg aca gag gtg ttc cgg gag gag ctg ggg gcc cgg cca   847
Asn Tyr Val Ala Thr Glu Val Phe Arg Glu Glu Leu Gly Ala Arg Pro
        215                 220                 225

gat gcc acc aaa gtg ctt atc atc atc acg gat ggg gag gcc act gac   895
Asp Ala Thr Lys Val Leu Ile Ile Ile Thr Asp Gly Glu Ala Thr Asp
    230                 235                 240

agt ggc aac atc gat gcg gcc aaa gac atc atc cgc tac atc atc ggg   943
Ser Gly Asn Ile Asp Ala Ala Lys Asp Ile Ile Arg Tyr Ile Ile Gly
245                 250                 255                 260

att gga aag cat ttt cag acc aag gag agt cag gag acc ctc cac aaa   991
Ile Gly Lys His Phe Gln Thr Lys Glu Ser Gln Glu Thr Leu His Lys
                265                 270                 275

ttt gca tca aaa ccc gcg agc gag ttt gtg aaa att ctg gac aca ttt  1039
Phe Ala Ser Lys Pro Ala Ser Glu Phe Val Lys Ile Leu Asp Thr Phe
            280                 285                 290

gag aag ctg aaa gat cta ttc act gag ctg cag aag aag atc tat gtc  1087
Glu Lys Leu Lys Asp Leu Phe Thr Glu Leu Gln Lys Lys Ile Tyr Val
        295                 300                 305

att gag ggc aca agc aaa cag gac ctg act tcc ttc aac atg gag ctg  1135
Ile Glu Gly Thr Ser Lys Gln Asp Leu Thr Ser Phe Asn Met Glu Leu
    310                 315                 320

tcc tcc agc ggc atc agt gct gac ctc agc agg ggc cat gca gtc gtg  1183
Ser Ser Ser Gly Ile Ser Ala Asp Leu Ser Arg Gly His Ala Val Val
325                 330                 335                 340

ggg gca gta gga gcc aag gac tgg gct ggg ggc ttt ctt gac ctg aag  1231
Gly Ala Val Gly Ala Lys Asp Trp Ala Gly Gly Phe Leu Asp Leu Lys
```

```
                345                 350                 355

gca gac ctg cag gat gac aca ttt att ggg aat gaa cca ttg aca cca   1279
Ala Asp Leu Gln Asp Asp Thr Phe Ile Gly Asn Glu Pro Leu Thr Pro
            360                 365                 370

gaa gtg aga gca ggc tat ttg ggt tac acc gtg acc tgg ctg ccc tcc   1327
Glu Val Arg Ala Gly Tyr Leu Gly Tyr Thr Val Thr Trp Leu Pro Ser
        375                 380                 385

cgg caa aag act tcg ttg ctg gcc tcg gga gcc cct cga tac cag cac   1375
Arg Gln Lys Thr Ser Leu Leu Ala Ser Gly Ala Pro Arg Tyr Gln His
    390                 395                 400

atg ggc cga gtg ctg ctg ttc caa gag cca cag ggc gga gga cac tgg   1423
Met Gly Arg Val Leu Leu Phe Gln Glu Pro Gln Gly Gly Gly His Trp
405                 410                 415                 420

agc cag gtc cag aca atc cat ggg acc cag att ggc tct tat ttc ggt   1471
Ser Gln Val Gln Thr Ile His Gly Thr Gln Ile Gly Ser Tyr Phe Gly
                425                 430                 435

ggg gag ctg tgt ggc gtc gac gtg gac caa gat ggg gag aca gag ctg   1519
Gly Glu Leu Cys Gly Val Asp Val Asp Gln Asp Gly Glu Thr Glu Leu
            440                 445                 450

ctg ctg att ggt gcc cca ctg ttc tat ggg gag cag aga gga ggc cgg   1567
Leu Leu Ile Gly Ala Pro Leu Phe Tyr Gly Glu Gln Arg Gly Gly Arg
        455                 460                 465

gtg ttt atc tac cag aga aga cag ttg ggg ttt gaa gaa gtc tca gag   1615
Val Phe Ile Tyr Gln Arg Arg Gln Leu Gly Phe Glu Glu Val Ser Glu
    470                 475                 480

ctg cag ggg gac ccc ggc tac cca ctc ggg cgg ttt gga gaa gcc atc   1663
Leu Gln Gly Asp Pro Gly Tyr Pro Leu Gly Arg Phe Gly Glu Ala Ile
485                 490                 495                 500

act gct ctg aca gac atc aac ggc gat ggg ctg gta gac gtg gct gtg   1711
Thr Ala Leu Thr Asp Ile Asn Gly Asp Gly Leu Val Asp Val Ala Val
                505                 510                 515

ggg gcc cct ctg gag gag cag ggg gct gtg tac atc ttc aat ggg agg   1759
Gly Ala Pro Leu Glu Glu Gln Gly Ala Val Tyr Ile Phe Asn Gly Arg
            520                 525                 530

cac ggg ggg ctt agt ccc cag cca agt cag cgg ata gaa ggg acc caa   1807
His Gly Gly Leu Ser Pro Gln Pro Ser Gln Arg Ile Glu Gly Thr Gln
        535                 540                 545

gtg ctc tca gga att cag tgg ttt gga cgc tcc atc cat ggg gtg aag   1855
Val Leu Ser Gly Ile Gln Trp Phe Gly Arg Ser Ile His Gly Val Lys
    550                 555                 560

gac ctt gaa ggg gat ggc ttg gca gat gtg gct gtg ggg gct gag agc   1903
Asp Leu Glu Gly Asp Gly Leu Ala Asp Val Ala Val Gly Ala Glu Ser
565                 570                 575                 580

cag atg atc gtg ctg agc tcc cgg ccc gtg gtg gat atg gtc acc ctg   1951
Gln Met Ile Val Leu Ser Ser Arg Pro Val Val Asp Met Val Thr Leu
                585                 590                 595
```

```
atg tcc ttc tct cca gct gag atc cca gtg cat gaa gtg gag tgc tcc   1999
Met Ser Phe Ser Pro Ala Glu Ile Pro Val His Glu Val Glu Cys Ser
600                 605                 610

tat tca acc agt aac aag atg aaa gaa gga gtt aat atc aca atc tgt   2047
Tyr Ser Thr Ser Asn Lys Met Lys Glu Gly Val Asn Ile Thr Ile Cys
615                 620                 625

ttc cag atc aag tct ctc tac ccc cag ttc caa ggc cgc ctg gtt gcc   2095
Phe Gln Ile Lys Ser Leu Tyr Pro Gln Phe Gln Gly Arg Leu Val Ala
630                 635                 640

aat ctc act tac act ctg cag ctg gat ggc cac cgg acc aga aga cgg   2143
Asn Leu Thr Tyr Thr Leu Gln Leu Asp Gly His Arg Thr Arg Arg Arg
645                 650                 655                 660

ggg ttg ttc cca gga ggg aga cat gaa ctc aga agg aat ata gct gtc   2191
Gly Leu Phe Pro Gly Gly Arg His Glu Leu Arg Arg Asn Ile Ala Val
665                 670                 675

acc acc agc atg tca tgc act gac ttc tca ttt cat ttc ccg gta tgt   2239
Thr Thr Ser Met Ser Cys Thr Asp Phe Ser Phe His Phe Pro Val Cys
680                 685                 690

gtt caa gac ctc atc tcc ccc atc aat gtt tcc ctg aat ttc tct ctt   2287
Val Gln Asp Leu Ile Ser Pro Ile Asn Val Ser Leu Asn Phe Ser Leu
695                 700                 705

tgg gag gag gaa ggg aca ccg agg gac caa agg gcg cag ggc aag gac   2335
Trp Glu Glu Glu Gly Thr Pro Arg Asp Gln Arg Ala Gln Gly Lys Asp
710                 715                 720

ata ccg ccc atc ctg aga ccc tcc ctg cac tcg gaa acc tgg gag atc   2383
Ile Pro Pro Ile Leu Arg Pro Ser Leu His Ser Glu Thr Trp Glu Ile
725                 730                 735                 740

cct ttt gag aag aac tgt ggg gag gac aag aag tgt gag gca aac ttg   2431
Pro Phe Glu Lys Asn Cys Gly Glu Asp Lys Lys Cys Glu Ala Asn Leu
745                 750                 755

aga gtg tcc ttc tct cct gca aga tcc aga gcc ctg cgt cta act gct   2479
Arg Val Ser Phe Ser Pro Ala Arg Ser Arg Ala Leu Arg Leu Thr Ala
760                 765                 770

ttt gcc agc ctc tct gtg gag ctg agc ctg agt aac ttg gaa gaa gat   2527
Phe Ala Ser Leu Ser Val Glu Leu Ser Leu Ser Asn Leu Glu Glu Asp
775                 780                 785

gct tac tgg gtc cag ctg gac ctg cac ttc ccc ccg gga ctc tcc ttc   2575
Ala Tyr Trp Val Gln Leu Asp Leu His Phe Pro Pro Gly Leu Ser Phe
790                 795                 800

cgc aag gtg gag atg ctg aag ccc cat agc cag ata cct gtg agc tgc   2623
Arg Lys Val Glu Met Leu Lys Pro His Ser Gln Ile Pro Val Ser Cys
805                 810                 815                 820

gag gag ctt cct gaa gag tcc agg ctt ctg tcc agg gca tta tct tgc   2671
Glu Glu Leu Pro Glu Glu Ser Arg Leu Leu Ser Arg Ala Leu Ser Cys
825                 830                 835

aat gtg agc tct ccc atc ttc aaa gca ggc cac tcg gtt gct ctg cag   2719
Asn Val Ser Ser Pro Ile Phe Lys Ala Gly His Ser Val Ala Leu Gln
```

```
            840                 845                 850

atg atg ttt aat aca ctg gta aac agc tcc tgg ggg gac tcg gtt gaa   2767
Met Met Phe Asn Thr Leu Val Asn Ser Ser Trp Gly Asp Ser Val Glu
        855                 860                 865

ttg cac gcc aat gtg acc tgt aac aat gag gac tca gac ctc ctg gag   2815
Leu His Ala Asn Val Thr Cys Asn Asn Glu Asp Ser Asp Leu Leu Glu
    870                 875                 880

gac aac tca gcc act acc atc atc ccc atc ctg tac ccc atc aac atc   2863
Asp Asn Ser Ala Thr Thr Ile Ile Pro Ile Leu Tyr Pro Ile Asn Ile
885                 890                 895                 900

ctc atc cag gac caa gaa gac tcc aca ctc tat gtc agt ttc acc ccc   2911
Leu Ile Gln Asp Gln Glu Asp Ser Thr Leu Tyr Val Ser Phe Thr Pro
                905                 910                 915

aaa ggc ccc aag atc cac caa gtc aag cac atg tac cag gtg agg atc   2959
Lys Gly Pro Lys Ile His Gln Val Lys His Met Tyr Gln Val Arg Ile
            920                 925                 930

cag cct tcc atc cac gac cac aac ata ccc acc ctg gag gct gtg gtt   3007
Gln Pro Ser Ile His Asp His Asn Ile Pro Thr Leu Glu Ala Val Val
        935                 940                 945

ggg gtg cca cag cct ccc agc gag ggg ccc atc aca cac cag tgg agc   3055
Gly Val Pro Gln Pro Pro Ser Glu Gly Pro Ile Thr His Gln Trp Ser
    950                 955                 960

gtg cag atg gag cct ccc gtg ccc tgc cac tat gag gat ctg gag agg   3103
Val Gln Met Glu Pro Pro Val Pro Cys His Tyr Glu Asp Leu Glu Arg
965                 970                 975                 980

ctc ccg gat gca gct gag cct tgt ctc ccc gga gcc ctg ttc cgc tgc   3151
Leu Pro Asp Ala Ala Glu Pro Cys Leu Pro Gly Ala Leu Phe Arg Cys
                985                 990                 995

cct gtt gtc ttc agg cag gag atc ctc gtc caa gtg atc ggg act ctg   3199
Pro Val Val Phe Arg Gln Glu Ile Leu Val Gln Val Ile Gly Thr Leu
            1000                1005                1010

gag ctg gtg gga gag atc gag gcc tct tcc atg ttc agc ctc tgc agc   3247
Glu Leu Val Gly Glu Ile Glu Ala Ser Ser Met Phe Ser Leu Cys Ser
        1015                1020                1025

tcc ctc tcc atc tcc ttc aac agc agc aag cat ttc cac ctc tat ggc   3295
Ser Leu Ser Ile Ser Phe Asn Ser Ser Lys His Phe His Leu Tyr Gly
    1030                1035                1040

agc aac gcc tcc ctg gcc cag gtt gtc atg aag gtt gac gtg gtg tat   3343
Ser Asn Ala Ser Leu Ala Gln Val Val Met Lys Val Asp Val Val Tyr
1045                1050                1055                1060

gag aag cag atg ctc tac ctc tac gtg ctg agc ggc atc ggg ggg ctg   3391
Glu Lys Gln Met Leu Tyr Leu Tyr Val Leu Ser Gly Ile Gly Gly Leu
                1065                1070                1075

ctg ctg ctg ctg ctc att ttc ata gtg ctg tac aag gtt ggt ttc ttc   3439
Leu Leu Leu Leu Leu Ile Phe Ile Val Leu Tyr Lys Val Gly Phe Phe
            1080                1085                1090
```

```
aaa cgg aac ctg aag gag aag atg gag gct ggc aga ggt gtc ccg aat   3487
Lys Arg Asn Leu Lys Glu Lys Met Glu Ala Gly Arg Gly Val Pro Asn
       1095                1100                1105

gga atc cct gca gaa gac tct gag cag ctg gca tct ggg caa gag gct   3535
Gly Ile Pro Ala Glu Asp Ser Glu Gln Leu Ala Ser Gly Gln Glu Ala
   1110                1115                1120

ggg gat ccc ggc tgc ctg aag ccc ctc cat gag aag gac tct gag agt   3583
Gly Asp Pro Gly Cys Leu Lys Pro Leu His Glu Lys Asp Ser Glu Ser
1125                1130                1135                1140

ggt ggt ggc aag gac tgagtccagg cctgtgaggt gcagagtgcc cagaactgga   3638
Gly Gly Gly Lys Asp
               1145

ctcaggatgc ccagggccac tctgcctctg cctgcattct gccgtgtgcc ctcgggcgag 3698

tcactgcctc tccctggccc tcagtttccc tatctcgaac atggaactca ttcctgaatg 3758

tctcctttgc aggctcatag ggaagacctg ctgagggacc agccaagagg gctgcaaaag 3818

tgagggcttg tcattaccag acggttcacc agcctctctt ggttccttcc ttggaagaga 3878

atgtctgatc taaatgtgga gaaactgtag tctcaggacc tagggatgtt ctggccctca 3938

cccctgccct gggatgtcca cagatgcctc cacccccag aacctgtcct tgcacactcc 3998

cctgcactgg agtccagtct cttctgctgg cagaaagcaa atgtgacctg tgtcactacg 4058

tgactgtggc acacgccttg ttcttggcca aagaccaaat tccttggcat gccttccagc 4118

accctgcaaa atgagaccct cgtggccttc cccagcctct tctagagccg tgatgcctcc 4178

ctgttgaagc tctggtgaca ccagcctttc tcccaggcca ggctccttcc tgtcttcctg 4238

cattcaccca gacagctccc tctgcctgaa ccttccatct cgcccacccc tccttccttg 4298

accagcagat cccagctcac gtcacacact tggttgggtc ctcacatctt tcacacttcc 4358

accaccctgc actactccct caaagcacac gtcatgtttc ttcatccggc agcctggatg 4418

ttttttccct gtttaatgat tgacgtactt agcagctatc tctcagtgaa ctgtgagggt 4478

aaaggctata cttgtcttgt tcaccttggg atgacgccgc atgatatgtc agggcgtggg 4538

acatctagta ggtgcttgac ataatttcac tgaattaatg acagagccag tgggaagata 4598

cagaaaaaga gggccggggc tgggcgcggt ggttcacgcc tgtaatccca gcactttggg 4658

aggccaagga gggtggatca cctgaggtca ggagttagag gccagcctgg cgaaacccca 4718

tctctactaa aaatacaaaa tccaggcgtg gtggcacaca cctgtagtcc cagctactca 4778

ggaggttgag gtaggagaat tgcttgaacc tgggaggtgg aggttgcagt gagccaagat 4838

tgcgccattg cactccagcc tgggcaacac agcgagactc cgtctcaagg aaaaaataaa 4898

aataaaaagc gggcacgggc ccggacatcc ccacccttgg aggctgtctt ctcaggctct 4958

gccctgccct agctccacac cctctcccag gacccatcac gcctgtgcag tggcccccac 5018
```

agaaagactg agctcaaggt gggaaccacg tctgctaact tggagcccca gtgccaagca 5078

cagtgcctgc atgtatttat ccaataaatg tgaaattctg tccaaaaaaa aaaaa 5133

<210> 2
<211> 1145
<212> PRT
<213> Homo sapiens

<400> 2

Tyr Asn Leu Asp Val Arg Gly Ala Arg Ser Phe Ser Pro Pro Arg Ala
1 5 10 15

Gly Arg His Phe Gly Tyr Arg Val Leu Gln Val Gly Asn Gly Val Ile
20 25 30

Val Gly Ala Pro Gly Glu Gly Asn Ser Thr Gly Ser Leu Tyr Gln Cys
35 40 45

Gln Ser Gly Thr Gly His Cys Leu Pro Val Thr Leu Arg Gly Ser Asn
50 55 60

Tyr Thr Ser Lys Tyr Leu Gly Met Thr Leu Ala Thr Asp Pro Thr Asp
65 70 75 80

Gly Ser Ile Leu Ala Cys Asp Pro Gly Leu Ser Arg Thr Cys Asp Gln
85 90 95

Asn Thr Tyr Leu Ser Gly Leu Cys Tyr Leu Phe Arg Gln Asn Leu Gln
100 105 110

Gly Pro Met Leu Gln Gly Arg Pro Gly Phe Gln Glu Cys Ile Lys Gly
115 120 125

Asn Val Asp Leu Val Phe Leu Phe Asp Gly Ser Met Ser Leu Gln Pro
130 135 140

Asp Glu Phe Gln Lys Ile Leu Asp Phe Met Lys Asp Val Met Lys Lys
145 150 155 160

Leu Ser Asn Thr Ser Tyr Gln Phe Ala Ala Val Gln Phe Ser Thr Ser
165 170 175

Tyr Lys Thr Glu Phe Asp Phe Ser Asp Tyr Val Lys Trp Lys Asp Pro
180 185 190

Asp Ala Leu Leu Lys His Val Lys His Met Leu Leu Leu Thr Asn Thr
195 200 205

Phe Gly Ala Ile Asn Tyr Val Ala Thr Glu Val Phe Arg Glu Glu Leu
210 215 220

Gly Ala Arg Pro Asp Ala Thr Lys Val Leu Ile Ile Ile Thr Asp Gly
225 230 235 240

Glu Ala Thr Asp Ser Gly Asn Ile Asp Ala Ala Lys Asp Ile Ile Arg
245 250 255

Tyr Ile Ile Gly Ile Gly Lys His Phe Gln Thr Lys Glu Ser Gln Glu
260 265 270

```
Thr Leu His Lys Phe Ala Ser Lys Pro Ala Ser Glu Phe Val Lys Ile
        275                 280                 285

Leu Asp Thr Phe Glu Lys Leu Lys Asp Leu Phe Thr Glu Leu Gln Lys
    290                 295                 300

Lys Ile Tyr Val Ile Glu Gly Thr Ser Lys Gln Asp Leu Thr Ser Phe
305                 310                 315                 320

Asn Met Glu Leu Ser Ser Ser Gly Ile Ser Ala Asp Leu Ser Arg Gly
                325                 330                 335

His Ala Val Val Gly Ala Val Gly Ala Lys Asp Trp Ala Gly Gly Phe
            340                 345                 350

Leu Asp Leu Lys Ala Asp Leu Gln Asp Asp Thr Phe Ile Gly Asn Glu
        355                 360                 365

Pro Leu Thr Pro Glu Val Arg Ala Gly Tyr Leu Gly Tyr Thr Val Thr
    370                 375                 380

Trp Leu Pro Ser Arg Gln Lys Thr Ser Leu Leu Ala Ser Gly Ala Pro
385                 390                 395                 400

Arg Tyr Gln His Met Gly Arg Val Leu Leu Phe Gln Glu Pro Gln Gly
                405                 410                 415

Gly Gly His Trp Ser Gln Val Gln Thr Ile His Gly Thr Gln Ile Gly
            420                 425                 430

Ser Tyr Phe Gly Gly Glu Leu Cys Gly Val Asp Val Asp Gln Asp Gly
        435                 440                 445

Glu Thr Glu Leu Leu Leu Ile Gly Ala Pro Leu Phe Tyr Gly Glu Gln
    450                 455                 460

Arg Gly Gly Arg Val Phe Ile Tyr Gln Arg Arg Gln Leu Gly Phe Glu
465                 470                 475                 480

Glu Val Ser Glu Leu Gln Gly Asp Pro Gly Tyr Pro Leu Gly Arg Phe
                485                 490                 495

Gly Glu Ala Ile Thr Ala Leu Thr Asp Ile Asn Gly Asp Gly Leu Val
            500                 505                 510

Asp Val Ala Val Gly Ala Pro Leu Glu Glu Gln Gly Ala Val Tyr Ile
        515                 520                 525

Phe Asn Gly Arg His Gly Gly Leu Ser Pro Gln Pro Ser Gln Arg Ile
    530                 535                 540

Glu Gly Thr Gln Val Leu Ser Gly Ile Gln Trp Phe Gly Arg Ser Ile
545                 550                 555                 560

His Gly Val Lys Asp Leu Glu Gly Asp Gly Leu Ala Asp Val Ala Val
                565                 570                 575

Gly Ala Glu Ser Gln Met Ile Val Leu Ser Ser Arg Pro Val Val Asp
            580                 585                 590

Met Val Thr Leu Met Ser Phe Ser Pro Ala Glu Ile Pro Val His Glu
        595                 600                 605
```

Val Glu Cys Ser Tyr Ser Thr Ser Asn Lys Met Lys Glu Gly Val Asn
610 615 620

Ile Thr Ile Cys Phe Gln Ile Lys Ser Leu Tyr Pro Gln Phe Gln Gly
625 630 635 640

Arg Leu Val Ala Asn Leu Thr Tyr Thr Leu Gln Leu Asp Gly His Arg
645 650 655

Thr Arg Arg Arg Gly Leu Phe Pro Gly Gly Arg His Glu Leu Arg Arg
660 665 670

Asn Ile Ala Val Thr Thr Ser Met Ser Cys Thr Asp Phe Ser Phe His
675 680 685

Phe Pro Val Cys Val Gln Asp Leu Ile Ser Pro Ile Asn Val Ser Leu
690 695 700

Asn Phe Ser Leu Trp Glu Glu Glu Gly Thr Pro Arg Asp Gln Arg Ala
705 710 715 720

Gln Gly Lys Asp Ile Pro Pro Ile Leu Arg Pro Ser Leu His Ser Glu
725 730 735

Thr Trp Glu Ile Pro Phe Glu Lys Asn Cys Gly Glu Asp Lys Lys Cys
740 745 750

Glu Ala Asn Leu Arg Val Ser Phe Ser Pro Ala Arg Ser Arg Ala Leu
755 760 765

Arg Leu Thr Ala Phe Ala Ser Leu Ser Val Glu Leu Ser Leu Ser Asn
770 775 780

Leu Glu Glu Asp Ala Tyr Trp Val Gln Leu Asp Leu His Phe Pro Pro
785 790 795 800

Gly Leu Ser Phe Arg Lys Val Glu Met Leu Lys Pro His Ser Gln Ile
805 810 815

Pro Val Ser Cys Glu Glu Leu Pro Glu Glu Ser Arg Leu Leu Ser Arg
820 825 830

Ala Leu Ser Cys Asn Val Ser Ser Pro Ile Phe Lys Ala Gly His Ser
835 840 845

Val Ala Leu Gln Met Met Phe Asn Thr Leu Val Asn Ser Ser Trp Gly
850 855 860

Asp Ser Val Glu Leu His Ala Asn Val Thr Cys Asn Asn Glu Asp Ser
865 870 875 880

Asp Leu Leu Glu Asp Asn Ser Ala Thr Thr Ile Ile Pro Ile Leu Tyr
885 890 895

Pro Ile Asn Ile Leu Ile Gln Asp Gln Glu Asp Ser Thr Leu Tyr Val
900 905 910

Ser Phe Thr Pro Lys Gly Pro Lys Ile His Gln Val Lys His Met Tyr
915 920 925

Gln Val Arg Ile Gln Pro Ser Ile His Asp His Asn Ile Pro Thr Leu
930 935 940

Glu Ala Val Val Gly Val Pro Gln Pro Pro Ser Glu Gly Pro Ile Thr
945 950 955 960

His Gln Trp Ser Val Gln Met Glu Pro Pro Val Pro Cys His Tyr Glu
965 970 975

Asp Leu Glu Arg Leu Pro Asp Ala Ala Glu Pro Cys Leu Pro Gly Ala
980 985 990

Leu Phe Arg Cys Pro Val Val Phe Arg Gln Glu Ile Leu Val Gln Val
995 1000 1005

Ile Gly Thr Leu Glu Leu Val Gly Glu Ile Glu Ala Ser Ser Met Phe
1010 1015 1020

Ser Leu Cys Ser Ser Leu Ser Ile Ser Phe Asn Ser Ser Lys His Phe
025 1030 1035 1040

His Leu Tyr Gly Ser Asn Ala Ser Leu Ala Gln Val Val Met Lys Val
1045 1050 1055

Asp Val Val Tyr Glu Lys Gln Met Leu Tyr Leu Tyr Val Leu Ser Gly
1060 1065 1070

Ile Gly Gly Leu Leu Leu Leu Leu Leu Ile Phe Ile Val Leu Tyr Lys
1075 1080 1085

Val Gly Phe Phe Lys Arg Asn Leu Lys Glu Lys Met Glu Ala Gly Arg
1090 1095 1100

Gly Val Pro Asn Gly Ile Pro Ala Glu Asp Ser Glu Gln Leu Ala Ser
105 1110 1115 1120

Gly Gln Glu Ala Gly Asp Pro Gly Cys Leu Lys Pro Leu His Glu Lys
1125 1130 1135

Asp Ser Glu Ser Gly Gly Gly Lys Asp
1140 1145

<210> 3
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 3
cccaagcttc cgccgccacc atggctccca gcag 34

<210> 4
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 4
tgctctagac tggtgatggt gatggtgatg aaaggtctgg agctggtagg gg 52

<210> 5
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 5
ctggtatttc tgtttgcggg ttcgatgagc ttg 33

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 6
gacttcatga aggatgcgat gaaactcagc 30

<210> 7
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 7
gaaggatgtg atgaaggcgc tcagcaacac ttgc 34

<210> 8
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 8
caattatgtc gcgacagcgg tgttccggga ggag 34

<210> 9
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 9
gcccggccag atgccgcgaa agtgcttatc atc 33

<210> 10

<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 10
cggccagatg ccaccgcggt gcttatcatc atc 33

<210> 11
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 11
gccaccaaag tgcttgcgat catcacggat ggg 33

<210> 12
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 12
catcgctgcg gccaaagcga tcatccgcta catc 34

<210> 13
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 13
gcggccaaag acatcgcgcg ctacatcatc ggg 33

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 14
cacaaatttg catcagcgcc cgcgagcgag tttg 34

<210> 15
<211> 34
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 15
gcatcaaaac ccgcggcgga gtttgtgaaa attc 34

<210> 16
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 16
caaaacccgc gagcgcgttt gtgaaaattc tg 32

<210> 17
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 17
gcgagcgagt ttgtggcgat tctggacaca tttg 34

<210> 18
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 18
ctggacacat ttgaggcgct gaaagatcta ttc 33

<210> 19
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 19
gaaagatcta ttcactgaga tgcagaagaa gatc 34

<210> 20
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 20
ctattcacgt gagctggcga agaagatcta tgtc 34

<210> 21
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 21
ttcactgagc tgcaggcgaa gatctatgtc attg 34

<210> 22
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 22
cactgagctg cagaaggcga tctatgtcat tgag 34

<210> 23
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 23
gagctgcaga agaaggcgta tgtcattgag ggc 33

<210> 24
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 24
ctgcagaaga agatcgcggt cattgagggc aca 33

<210> 25
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 25
acggatgggg aggccgcgga cagtggcaac atc 33

<210> 26
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 26
ggggaggcca ctgacgcggg aaacatcgat gc 32

<210> 27
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 27
gccactgaca gtggcgcgat cgatgcggcc aaag 34

<210> 28
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 28
gacagtggca acatcgcggc ggccaaagac atc 33

<210> 29
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 29
caacatcgat gcggccgcgg acatcatccg ctac 34

<210> 30
<211> 2704
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(2307)

<400> 30

```
atg ctg ggc ctg cgc ccc cca ctg ctc gcc ctg gtg ggg ctg ctc tcc   48
Met Leu Gly Leu Arg Pro Pro Leu Leu Ala Leu Val Gly Leu Leu Ser
 1               5                  10                  15

ctc ggg tgc gtc ctc tct cag gag tgc acg aag ttc aag gtc agc agc   96
Leu Gly Cys Val Leu Ser Gln Glu Cys Thr Lys Phe Lys Val Ser Ser
            20                  25                  30

tgc cgg gaa tgc atc gag tcg ggg ccc ggc tgc acc tgg tgc cag aag   144
Cys Arg Glu Cys Ile Glu Ser Gly Pro Gly Cys Thr Trp Cys Gln Lys
        35                  40                  45

ctg aac ttc aca ggg ccg ggg gat cct gac tcc att cgc tgc gac acc   192
Leu Asn Phe Thr Gly Pro Gly Asp Pro Asp Ser Ile Arg Cys Asp Thr
    50                  55                  60

cgg cca cag ctg ctc atg agg ggc tgt gcg gct gac gac atc atg gac   240
Arg Pro Gln Leu Leu Met Arg Gly Cys Ala Ala Asp Asp Ile Met Asp
65                  70                  75                  80

ccc aca agc ctc gct gaa acc cag gaa gac cac aat ggg ggc cag aag   288
Pro Thr Ser Leu Ala Glu Thr Gln Glu Asp His Asn Gly Gly Gln Lys
                85                  90                  95

cag ctg tcc cca caa aaa gtg acg ctt tac ctg cga cca ggc cag gca   336
Gln Leu Ser Pro Gln Lys Val Thr Leu Tyr Leu Arg Pro Gly Gln Ala
            100                 105                 110

gca gcg ttc aac gtg acc ttc cgg cgg gcc aag ggc tac ccc atc gac   384
Ala Ala Phe Asn Val Thr Phe Arg Arg Ala Lys Gly Tyr Pro Ile Asp
        115                 120                 125

ctg tac tat ctg atg gac ctc tcc tac tcc atg ctt gat gac ctc agg   432
Leu Tyr Tyr Leu Met Asp Leu Ser Tyr Ser Met Leu Asp Asp Leu Arg
    130                 135                 140

aat gtc aag aag cta ggt ggc gac ctg ctc cgg gcc ctc aac gag atc   480
Asn Val Lys Lys Leu Gly Gly Asp Leu Leu Arg Ala Leu Asn Glu Ile
145                 150                 155                 160

acc gag tcc ggc cgc att ggc ttc ggg tcc ttc gtg gac aag acc gtg   528
Thr Glu Ser Gly Arg Ile Gly Phe Gly Ser Phe Val Asp Lys Thr Val
                165                 170                 175

ctg ccg ttc gtg aac acg cac cct gat aag ctg cga aac cca tgc ccc   576
Leu Pro Phe Val Asn Thr His Pro Asp Lys Leu Arg Asn Pro Cys Pro
            180                 185                 190

aac aag gag aaa gag tgc cag ccc ccg ttt gcc ttc agg cac gtg ctg   624
Asn Lys Glu Lys Glu Cys Gln Pro Pro Phe Ala Phe Arg His Val Leu
        195                 200                 205

aag ctg acc aac aac tcc aac cag ttt cag acc gag gtc ggg aag cag   672
Lys Leu Thr Asn Asn Ser Asn Gln Phe Gln Thr Glu Val Gly Lys Gln
    210                 215                 220

ctg att tcc gga aac ctg gat gca ccc gag ggt ggg ctg gac gcc atg   720
Leu Ile Ser Gly Asn Leu Asp Ala Pro Glu Gly Gly Leu Asp Ala Met
225                 230                 235                 240

atg cag gtc gcc gcc tgc ccg gag gaa atc ggc tgg cgc aac gtc acg   768
Met Gln Val Ala Ala Cys Pro Glu Glu Ile Gly Trp Arg Asn Val Thr
```

```
                245                 250                 255

cgg ctg ctg gtg ttt gcc act gat gac ggc ttc cat ttc gcg ggc gac   816
Arg Leu Leu Val Phe Ala Thr Asp Asp Gly Phe His Phe Ala Gly Asp
            260                 265                 270

gga aag ctg ggc gcc atc ctg acc ccc aac gac ggc cgc tgt cac ctg   864
Gly Lys Leu Gly Ala Ile Leu Thr Pro Asn Asp Gly Arg Cys His Leu
        275                 280                 285

gag gac aac ttg tac aag agg agc aac gaa ttc gac tac cca tcg gtg   912
Glu Asp Asn Leu Tyr Lys Arg Ser Asn Glu Phe Asp Tyr Pro Ser Val
    290                 295                 300

ggc cag ctg gcg cac aag ctg gct gaa aac aac atc cag ccc atc ttc   960
Gly Gln Leu Ala His Lys Leu Ala Glu Asn Asn Ile Gln Pro Ile Phe
305                 310                 315                 320

gcg gtg acc agt agg atg gtg aag acc tac gag aaa ctc acc gag atc  1008
Ala Val Thr Ser Arg Met Val Lys Thr Tyr Glu Lys Leu Thr Glu Ile
                325                 330                 335

atc ccc aag tca gcc gtg ggg gag ctg tct gag gac tcc agc aat gtg  1056
Ile Pro Lys Ser Ala Val Gly Glu Leu Ser Glu Asp Ser Ser Asn Val
            340                 345                 350

gtc cat ctc att aag aat gct tac aat aaa ctc tcc tcc agg gtc ttc  1104
Val His Leu Ile Lys Asn Ala Tyr Asn Lys Leu Ser Ser Arg Val Phe
        355                 360                 365

ctg gat cac aac gcc ctc ccc gac acc ctg aaa gtc acc tac gac tcc  1152
Leu Asp His Asn Ala Leu Pro Asp Thr Leu Lys Val Thr Tyr Asp Ser
    370                 375                 380

ttc tgc agc aat gga gtg acg cac agg aac cag ccc aga ggt gac tgt  1200
Phe Cys Ser Asn Gly Val Thr His Arg Asn Gln Pro Arg Gly Asp Cys
385                 390                 395                 400

gat ggc gtg cag atc aat gtc ccg atc acc ttc cag gtg aag gtc acg  1248
Asp Gly Val Gln Ile Asn Val Pro Ile Thr Phe Gln Val Lys Val Thr
                405                 410                 415

gcc aca gag tgc atc cag gag cag tcg ttt gtc atc cgg gcg ctg ggc  1296
Ala Thr Glu Cys Ile Gln Glu Gln Ser Phe Val Ile Arg Ala Leu Gly
            420                 425                 430

ttc acg gac ata gtg acc gtg cag gtt ctt ccc cag tgt gag tgc cgg  1344
Phe Thr Asp Ile Val Thr Val Gln Val Leu Pro Gln Cys Glu Cys Arg
        435                 440                 445

tgc cgg gac cag agc aga gac cgc agc ctc tgc cat ggc aag ggc ttc  1392
Cys Arg Asp Gln Ser Arg Asp Arg Ser Leu Cys His Gly Lys Gly Phe
    450                 455                 460

ttg gag tgc ggc atc tgc agg tgt gac act ggc tac att ggg aaa aac  1440
Leu Glu Cys Gly Ile Cys Arg Cys Asp Thr Gly Tyr Ile Gly Lys Asn
465                 470                 475                 480

tgt gag tgc cag aca cag ggc cgg agc agc cag gag ctg gaa gga agc  1488
Cys Glu Cys Gln Thr Gln Gly Arg Ser Ser Gln Glu Leu Glu Gly Ser
                485                 490                 495
```

```
tgc cgg aag gac aac aac tcc atc atc tgc tca ggg ctg ggg gac tgt   1536
Cys Arg Lys Asp Asn Asn Ser Ile Ile Cys Ser Gly Leu Gly Asp Cys
            500                 505                 510

gtc tgc ggg cag tgc ctg tgc cac acc agc gac gtc ccc ggc aag ctg   1584
Val Cys Gly Gln Cys Leu Cys His Thr Ser Asp Val Pro Gly Lys Leu
        515                 520                 525

ata tac ggg cag tac tgc gag tgt gac acc atc aac tgt gag cgc tac   1632
Ile Tyr Gly Gln Tyr Cys Glu Cys Asp Thr Ile Asn Cys Glu Arg Tyr
    530                 535                 540

aac ggc cag gtc tgc ggc ggc ccg ggg agg ggg ctc tgc ttc tgc ggg   1680
Asn Gly Gln Val Cys Gly Gly Pro Gly Arg Gly Leu Cys Phe Cys Gly
545                 550                 555                 560

aag tgc cgc tgc cac ccg ggc ttt gag ggc tca gcg tgc cag tgc gag   1728
Lys Cys Arg Cys His Pro Gly Phe Glu Gly Ser Ala Cys Gln Cys Glu
                565                 570                 575

agg acc act gag ggc tgc ctg aac ccg cgg cgt gtt gag tgt agt ggt   1776
Arg Thr Thr Glu Gly Cys Leu Asn Pro Arg Arg Val Glu Cys Ser Gly
            580                 585                 590

cgt ggc cgg tgc cgc tgc aac gta tgc gag tgc cat tca ggc tac cag   1824
Arg Gly Arg Cys Arg Cys Asn Val Cys Glu Cys His Ser Gly Tyr Gln
        595                 600                 605

ctg cct ctg tgc cag gag tgc ccc ggc tgc ccc tca ccc tgt ggc aag   1872
Leu Pro Leu Cys Gln Glu Cys Pro Gly Cys Pro Ser Pro Cys Gly Lys
    610                 615                 620

tac atc tcc tgc gcc gag tgc ctg aag ttc gaa aag ggc ccc ttt ggg   1920
Tyr Ile Ser Cys Ala Glu Cys Leu Lys Phe Glu Lys Gly Pro Phe Gly
625                 630                 635                 640

aag aac tgc agc gcg gcg tgt ccg ggc ctg cag ctg tcg aac aac ccc   1968
Lys Asn Cys Ser Ala Ala Cys Pro Gly Leu Gln Leu Ser Asn Asn Pro
                645                 650                 655

gtg aag ggc agg acc tgc aag gag agg gac tca gag ggc tgc tgg gtg   2016
Val Lys Gly Arg Thr Cys Lys Glu Arg Asp Ser Glu Gly Cys Trp Val
            660                 665                 670

gcc tac acg ctg gag cag cag gac ggg atg gac cgc tac ctc atc tat   2064
Ala Tyr Thr Leu Glu Gln Gln Asp Gly Met Asp Arg Tyr Leu Ile Tyr
        675                 680                 685

gtg gat gag agc cga gag tgt gtg gca ggc ccc aac atc gcc gcc atc   2112
Val Asp Glu Ser Arg Glu Cys Val Ala Gly Pro Asn Ile Ala Ala Ile
    690                 695                 700

gtc ggg ggc acc gtg gca ggc atc gtg ctg atc ggc att ctc ctg ctg   2160
Val Gly Gly Thr Val Ala Gly Ile Val Leu Ile Gly Ile Leu Leu Leu
705                 710                 715                 720

gtc atc tgg aag gct ctg atc cac ctg agc gac ctc cgg gag tac agg   2208
Val Ile Trp Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg
                725                 730                 735

cgc ttt gag aag gag aag ctc aag tcc cag tgg aac aat gat aat ccc   2256
Arg Phe Glu Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro
```

740 745 750

ctt ttc aag agc gcc acc acg acg gtc atg aac ccc aag ttt gct gag 2304
Leu Phe Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu
755 760 765

agt taggagcact tggtgaagac aaggccgtca ggacccacca tgtctgcccc 2357
Ser

atcacgcggc cgagacatgg cttggccaca gctcttgagg atgtcaccaa ttaaccagaa 2417

atccagttat tttccgccct caaaatgaca gccatggccg gccggtgctt ctgggggctc 2477

gtcgggggga cagctccact ctgactggca cagtctttgc atggagactt gaggagggct 2537

tgaggttggt gaggttaggt gcgtgtttcc tgtgcaagtc aggacatcag tctgattaaa 2597

ggtggtgcca atttatttac atttaaactt gtcagggtat aaaatgacat cccattaatt 2657

atattgttaa tcaatcacgt gtatagaaaa aaaaataaaa cttcaat 2704

<210> 31
<211> 769
<212> PRT
<213> Homo sapiens

<400> 31
Met Leu Gly Leu Arg Pro Pro Leu Leu Ala Leu Val Gly Leu Leu Ser
1 5 10 15

Leu Gly Cys Val Leu Ser Gln Glu Cys Thr Lys Phe Lys Val Ser Ser
20 25 30

Cys Arg Glu Cys Ile Glu Ser Gly Pro Gly Cys Thr Trp Cys Gln Lys
35 40 45

Leu Asn Phe Thr Gly Pro Gly Asp Pro Asp Ser Ile Arg Cys Asp Thr
50 55 60

Arg Pro Gln Leu Leu Met Arg Gly Cys Ala Ala Asp Asp Ile Met Asp
65 70 75 80

Pro Thr Ser Leu Ala Glu Thr Gln Glu Asp His Asn Gly Gly Gln Lys
85 90 95

Gln Leu Ser Pro Gln Lys Val Thr Leu Tyr Leu Arg Pro Gly Gln Ala
100 105 110

Ala Ala Phe Asn Val Thr Phe Arg Arg Ala Lys Gly Tyr Pro Ile Asp
115 120 125

Leu Tyr Tyr Leu Met Asp Leu Ser Tyr Ser Met Leu Asp Asp Leu Arg
130 135 140

Asn Val Lys Lys Leu Gly Gly Asp Leu Leu Arg Ala Leu Asn Glu Ile
145 150 155 160

Thr Glu Ser Gly Arg Ile Gly Phe Gly Ser Phe Val Asp Lys Thr Val
165 170 175

Leu Pro Phe Val Asn Thr His Pro Asp Lys Leu Arg Asn Pro Cys Pro

180 185 190

Asn Lys Glu Lys Glu Cys Gln Pro Pro Phe Ala Phe Arg His Val Leu
195 200 205

Lys Leu Thr Asn Asn Ser Asn Gln Phe Gln Thr Glu Val Gly Lys Gln
210 215 220

Leu Ile Ser Gly Asn Leu Asp Ala Pro Glu Gly Gly Leu Asp Ala Met
225 230 235 240

Met Gln Val Ala Ala Cys Pro Glu Glu Ile Gly Trp Arg Asn Val Thr
245 250 255

Arg Leu Leu Val Phe Ala Thr Asp Asp Gly Phe His Phe Ala Gly Asp
260 265 270

Gly Lys Leu Gly Ala Ile Leu Thr Pro Asn Asp Gly Arg Cys His Leu
275 280 285

Glu Asp Asn Leu Tyr Lys Arg Ser Asn Glu Phe Asp Tyr Pro Ser Val
290 295 300

Gly Gln Leu Ala His Lys Leu Ala Glu Asn Asn Ile Gln Pro Ile Phe
305 310 315 320

Ala Val Thr Ser Arg Met Val Lys Thr Tyr Glu Lys Leu Thr Glu Ile
325 330 335

Ile Pro Lys Ser Ala Val Gly Glu Leu Ser Glu Asp Ser Ser Asn Val
340 345 350

Val His Leu Ile Lys Asn Ala Tyr Asn Lys Leu Ser Ser Arg Val Phe
355 360 365

Leu Asp His Asn Ala Leu Pro Asp Thr Leu Lys Val Thr Tyr Asp Ser
370 375 380

Phe Cys Ser Asn Gly Val Thr His Arg Asn Gln Pro Arg Gly Asp Cys
385 390 395 400

Asp Gly Val Gln Ile Asn Val Pro Ile Thr Phe Gln Val Lys Val Thr
405 410 415

Ala Thr Glu Cys Ile Gln Glu Gln Ser Phe Val Ile Arg Ala Leu Gly
420 425 430

Phe Thr Asp Ile Val Thr Val Gln Val Leu Pro Gln Cys Glu Cys Arg
435 440 445

Cys Arg Asp Gln Ser Arg Asp Arg Ser Leu Cys His Gly Lys Gly Phe
450 455 460

Leu Glu Cys Gly Ile Cys Arg Cys Asp Thr Gly Tyr Ile Gly Lys Asn
465 470 475 480

Cys Glu Cys Gln Thr Gln Gly Arg Ser Ser Gln Glu Leu Glu Gly Ser
485 490 495

Cys Arg Lys Asp Asn Asn Ser Ile Ile Cys Ser Gly Leu Gly Asp Cys
500 505 510

Val Cys Gly Gln Cys Leu Cys His Thr Ser Asp Val Pro Gly Lys Leu
515 520 525

Ile Tyr Gly Gln Tyr Cys Glu Cys Asp Thr Ile Asn Cys Glu Arg Tyr
530 535 540

Asn Gly Gln Val Cys Gly Gly Pro Gly Arg Gly Leu Cys Phe Cys Gly
545 550 555 560

Lys Cys Arg Cys His Pro Gly Phe Glu Gly Ser Ala Cys Gln Cys Glu
565 570 575

Arg Thr Thr Glu Gly Cys Leu Asn Pro Arg Arg Val Glu Cys Ser Gly
580 585 590

Arg Gly Arg Cys Arg Cys Asn Val Cys Glu Cys His Ser Gly Tyr Gln
595 600 605

Leu Pro Leu Cys Gln Glu Cys Pro Gly Cys Pro Ser Pro Cys Gly Lys
610 615 620

Tyr Ile Ser Cys Ala Glu Cys Leu Lys Phe Glu Lys Gly Pro Phe Gly
625 630 635 640

Lys Asn Cys Ser Ala Ala Cys Pro Gly Leu Gln Leu Ser Asn Asn Pro
645 650 655

Val Lys Gly Arg Thr Cys Lys Glu Arg Asp Ser Glu Gly Cys Trp Val
660 665 670

Ala Tyr Thr Leu Glu Gln Gln Asp Gly Met Asp Arg Tyr Leu Ile Tyr
675 680 685

Val Asp Glu Ser Arg Glu Cys Val Ala Gly Pro Asn Ile Ala Ala Ile
690 695 700

Val Gly Gly Thr Val Ala Gly Ile Val Leu Ile Gly Ile Leu Leu Leu
705 710 715 720

Val Ile Trp Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg
725 730 735

Arg Phe Glu Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro
740 745 750

Leu Phe Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu
755 760 765

Ser

<210> 32
<211> 47
<212> PRT
<213> Homo sapiens

<400> 32
Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro
1 5 10 15

Ser Ala Gln Leu Glu Lys Glu Leu Gln Ala Leu Glu Lys Glu Asn Ala

20 25 30

Gln Leu Glu Trp Glu Leu Gln Ala Leu Glu Lys Glu Leu Ala Gln
35 40 45

<210> 33
<211> 47
<212> PRT
<213> Homo sapiens

<400> 33
Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro
1 5 10 15

Ser Ala Gln Leu Lys Lys Lys Leu Gln Ala Leu Lys Lys Lys Asn Ala
20 25 30

Gln Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala Gln
35 40 45